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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Sudhir	PAUL	Missouri City, Texas

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

COVALENT ATTACHMENT OF LIGANDS TO NUCLEOPHILIC PROTEINS GUIDED BY NON-COVALENT BINDING

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 8

☑ Drawing(s) *Number of Sheets* 5

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☑ Other (specify) Attachments A-D (26 pages)

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Respectfully submitted,

SIGNATURE _____ Date 03/26/2003

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REGISTRATION NO. *(if appropriate)* 33,893

Docket Number: 330199.00200

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Provisional Application

Covalent Attachment of Ligands to Nucleophilic Proteins Guided by Non-Covalent Binding

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Introduction

Non-catalytic proteins display nucleophilic reactivity upon specific binding to electrophillic compounds of formula (1) and (2). These activated nucleophiles function in register with conventional noncovalent binding sites in the protein. Until now, such activated nucleophiles were thought to exist only in the catalytic sites of enzymes, such as those found in serine proteases. In the present invention, nucleophilic sites have been identified in several non-enzymatic proteins, including albumin, gp120 and antibodies directed to gp120 and EGFR. The presence of activated nucleophiles in these proteins indicates the formation of specific non-covalent bonding interactions with the inventive compounds that arise from protein folding and protein interactions with other macromolecules and small molecules. Biotin-containing and fluorescent phosphonate esters are employed to identify the nucleophilic sites. Non-covalent binding to moieties in the spatial neighborhood of the phosphonate electrophile allows targeting of nucleophiles in proteins with defined binding specificity (Fig 1; see also Fig 2 for examples of specific structures that are the subject of this invention). Such nucleophilic proteins are designated nucleophilic receptors (NURs) for the purpose of the present invention. Electrophilic covalently reactive analogs of ligands capable of covalent binding to NURs guided by noncovalent interactions are designated CALs (Covalent Analogues of Ligands) for the purpose of the present invention. In the extreme case, CALs of NURs can be prepared (designated CALNURs), allowing a given molecule of the CALNUR expressing to a nucleophile to bind covalently to a second CALNUR molecule at the covalently reactive electrophile incorporated within the CALNUR.

The invention can be used for (a) irreversible inhibition of pathogenic NUR activities using CALs; (b) formation of covalently bonded, stable mimics of non-covalent, supramolecular CALNURs complexes with correspondingly stabilized biological activity; (c) facile isolation of

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individual NUR polypeptides and their genes from libraries displayed on the surface of viruses, prokaryotic cells and eukaryotic cells, including NURs capable of covalent binding to natural, unmodified macromolecules and small molecules; (d) directed evolution of NURs expressing the desired binding activity by random or directed sequence diversification followed by selection for active site nucleophiles in register with the non-covalent binding site.

Background

Activated nucleophilic residues in conventional serine proteases are known to react covalently with phosphonate diester probes, e.g., the Ser residue in the catalytic Ser-His-Asp triad of serine proteases. Such nucleophiles have also been observed in proteolytic and esterolytic antibodies (Abs) from mutagenesis and covalent phosphonate binding studies. Nucleophilic attack on the substrate is the rate limiting step in catalysis by certain enzymes. Since the reported catalytic rate constants (kcat) of antibodies are generally orders of magnitude lower than enzymes, it has generally been assumed that the deficiency resides in the poor nucleophilic reactivity of Abs. The first step underlying the present invention was the realization that non-catalytic Abs and other non-catalytic proteins are not deficient in nucleophilic reactivity. .

We have found that despite their low proteolytic activity, IgG preparations can display stronger nucleophilic reactivity than trypsin, as determined from rates of formation of covalent adducts with hapten phosphonate diesters of the present invention. Studies of polyclonal IgG and individual scFv clones indicate there is an apparently universal nucleophilic reactivity in what are normally regarded as being non-catalytic proteins. In control experiments this reactivity was lost upon thermal denaturation of the proteins, consistent with the idea that activation of the nucleophile depends on the native protein structure. Also, covalent binding of the antibodies to the phosphonate diester probe was inhibited by the known serine protease-reactive reagent, DFP. Additional proteins identified as expressing nucleophilic reactivity are the HIV coat protein gp120, and bovine serum albumin, and also the polypeptide vasoactive intestinal peptide.

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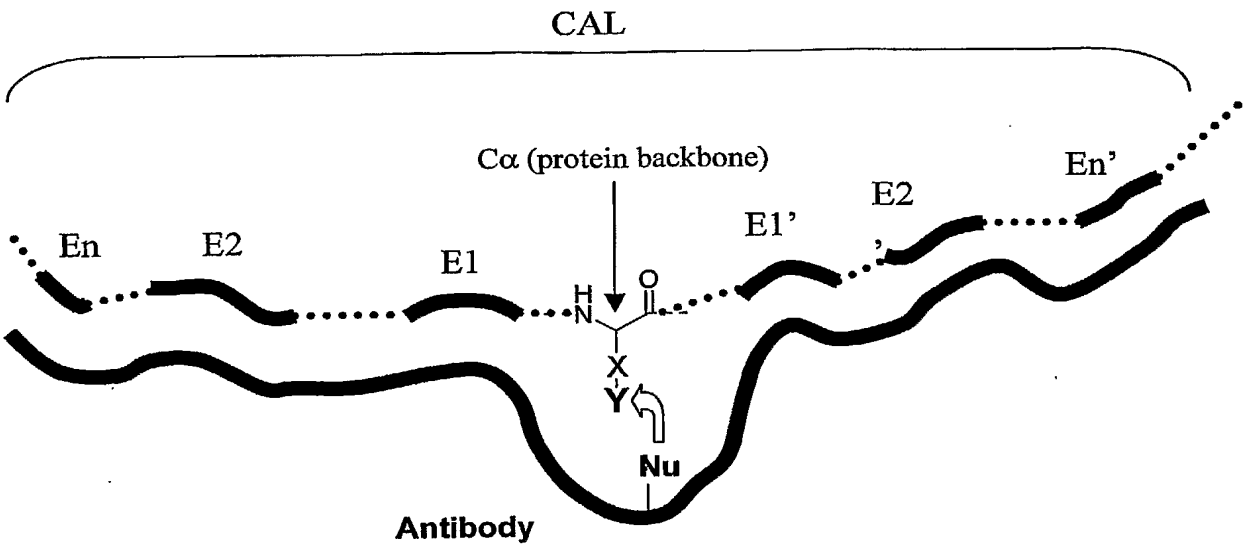

Fig 1: Covalent analogs of ligands (CALs) can be derivatives of proteins and polypeptides in which one or more amino acid side chains are linked to the electrophilic group (Y, e.g. phosphonate ester). Linkage of Y to the amino acid side chain can be accomplished directly or through the use of an adaptor functionality, which is then considered to be a component of Y in the general pCRA formula shown here. X corresponds to the side chain of any amino acid present in the parent protein. Typical examples of X are the side chains of Lys, Asp, Glu, Cys, Ser, Thr and Tyr. Examples of the site of linkage of Y to these amino acids include the –NH2, -COOH, -SH and -OH groups. E1, E2, ···En and E1', E2', ··· En' are the component amino acids of the antigenic epitope recognized noncovalently by the antibody. The noncovalent interactions occur in conjunction with covalent interaction between one or more antibody nucleophile (Nu) and one or more CAL electrophile Y. E1-En and E-'-En' can be a linear or discontinuous set of amino acids that are spatially in proximity with electrophile Y. Dotted lines connecting E1-En and E'-En' can represent short or extended lengths of the polypeptide backbone that do not serve as components of the antigenic epitope. As proteins can express one or more antigenic epitope, the CAL may contain one or more sets of each reactive unit composed of E1-En, E1'-En', X and Y.

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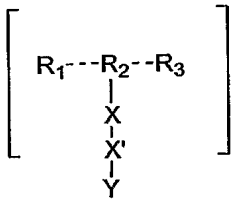
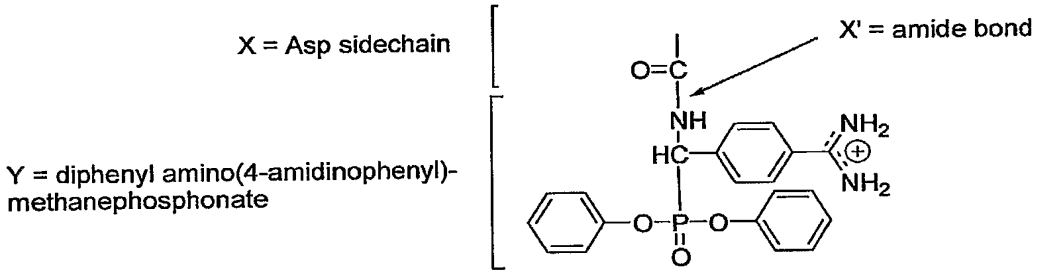


General CAL structure

X-X'-Y, Example 1:



X-X'-Y, Example 2

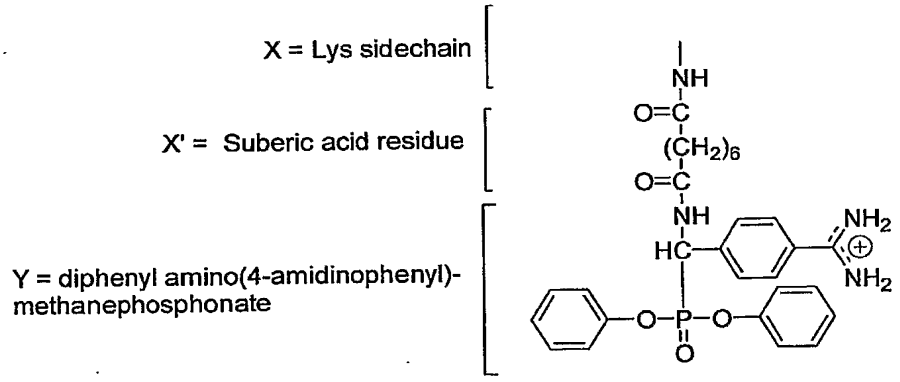


Fig 2. General CAL structure and examples of its components

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Nucleophilic proteins that react with electrophilic compounds disclosed here can be identified by methods in which genetic repertoire of the proteins are cloned into the phage genome, the repertoires are displayed on the surface of phages, and covalent binding by the electrophilic compounds is applied as the means to separate the desires phages. The proteins are often cloned as fragments that can be recloned as full-length proteins once fragments with the desired nucleophilic activity has been identified (e.g., US patents 6,407,213 and 5,807,715). Improvements in the activity can be achieved by mutagenesis of the genes and identification of the mutant proteins with the highest nucleophilic activity (e.g, US patents 5,811,238 and 6,406,863).

The manuscript attached hereto entitled *Broadly Distributed Chemical Reactivity of Natural Antibodies Expressed in Coordination with Specific Antigen Binding Activity* (Planque et al.) and its Figures 1 - 7 are part of this provisional application. The following documents and their associated figures, as referenced within the documents, are also parts of this provisional application:

a) Pathogenic Antibodies to DNA and VIP; Appendix Figs. A – E.

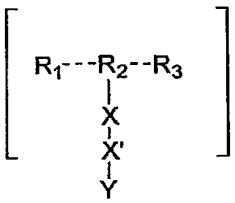
b) Covalently reactive CD4 Analogs.

c) Covalent gp120 Oligomers for Vaccination and Antibody Generation

d) Examples of sites of attachment of electrophile Y to peptdes and proteins.

Claims

1. A compound of formula (1)



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wherein,

Y is a covalently reactive electrophilic group, X' is a chemical bond or adaptor group, X is a functional group of R2 to which the adaptor is attached, R2 is a component unit of the ligand such as an amino acid, sugar residue or fatty acid group, and R1 and R3 are chemical groups that are located in the spatial neighborhood of Y and contribute one or more sites capable of specific noncovalent binding to a protein.

2. The compound of claim 1, wherein Y is a phosphonate ester.

3. The compound of claim 1 in which Y is a carbonyl group such as the carbonyl group found in pyruvic acid.

4. The compound of claim 1 in which X' is an adaptor molecule such as but not limited to suberic acid and 6-aminocaproic acid.

5. The compound of claim 1 in which X is an amino acid side chain containing a reactive group to which X'-Y can be attached, such as but not limited to lysine side chains containing an amine group

6. The compound of claim 1 in which R2 is the alpha carbon atom of an amino acid located in the backbone of a peptide or protein ligand.

7. The compound of claim 1 in which R1 and R3 are noncovalently reactive functional groups usually located within a sphere of radius of 50 angstroms with Y at its center.

8. The compound of claim 1 in which R1 and R3 are one or more amino acid components of a protein or peptide located within a sphere of radius of 50 angstroms with Y at its center and are capable of noncovalent binding to other proteins.

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9. The compound of claim 1 in which R1 and R3 are one or more noncontiguous or contiguous components of the peptide Cys-Lys-Gln-Ile-Ile-Asn-Met-Trp-Gln-Glu-Val- , R2 is Gly, X is the carboxyl group of Gly, X' is an amide bond, Y is diphenyl amino(4-amidinophenyl)methanephosphonate.

10. The compound of claim 1 wherein R_1 and R3 are amino acids belonging to EGFR.

11. The compound of claim 1 wherein R1 and R3 are amino acids belonging to gp120.

12. The compound of claim 1 wherein R1 and R3 are amino acids belonging to vasoactive intestinal peptide.

13. The compound of claim 1, wherein R_1 or R3 contains a biotin moiety, a fluorescent moiety, a radioisotope or a toxin .

14. The compound of claim 1 wherein X' contains a biotin moiety, a fluorescent moiety, a radioisotope or a toxin.

15. The compound of claim 1 wherein Y is composed of an electrophile attached to a biotin moiety, fluorescent moiety, a radioisotope or a toxin.

16. A method of altering the magnitude and nature of the activity of a target protein, comprising: contacting a compound of formula (1) in claim 1 with a target protein that binds specifically to said compound of formula (1) and which has a nucleophillic residue, thereby forming a covalent bond with said nucleophillic residue.

17. The method of claim 16 in which the protein activity is inhibited by contact with a compound of formula (1).

18. The method of claim 16 in which the protein activity is stimulated by contact with a compound of formula (1).

19. The method of claim 16, wherein the target protein is produced by a microbe, tumor cells, normal animal cells or normal human cells.

20. The method of claim 16 in which the target protein is necessary for survival and growth of a microbe, a protein that serves as a growth promoter of animal cells including human

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cells, a protein that promotes tumor metastasis, an endogenous animal or human protein, a protein that promotes inflammation, a protein that inhibits inflammation, a protein that promotes blood coagulation, a protein that inhibits blood coagulation, a neurotramitter, a hormone, a cell surface receptor protein, a protein that transports a metabolite or a drug, a protein that is essential for immunological defense, and an autoantibody.

21. The method of claim 16, wherein the target protein is gp120, CD4, epidermal growth factor receptor, epidermal growth factor, vasoactive intestinal peptide, receptors for vasoactive intestinal peptide, a cytokine, a receptor for a cytokine, prothrombin, Factor Xa, Factor VIII, albumin, an autoantibody to DNA, an autoantibody to vasoactive intestinal peptide, an autoantibody to insulin, an autoantibody to an insulin receptor, an autoantibody to the acetylcholine receptor .

22. A method for synthesis of stable covalent mimics of natural homo-oligomer molecular assemblies consisting of spontaneous noncovalent association of a compound with general formula (1) in claim 1 followed by covalent attachment of electrophile Y with a naturally occurring nucleophile in the compound.

23. The method of claim 22 in which the self-associating molecule is gp120 with general formula (1) in claim 1.

24. The method of claim 22 in which the self associating molecule is a animal or human protein including but not limited to proteins that form ion channels in lipid membranes.

25. The method of claim 22 in which the covalent mimetic is employed as a prophylactic vaccine.

26. The method of claim 22 in which the covalent mimetic is employed as immunogen to elicit the synthesis of monoclonal antibodies.

27. The method of claim 22 in which the covalent mimetic is employed to identify and isolate nucleophilic proteins and their genes from displayed molecular libraries.

28. The method of claim 27 in which the displayed libraries are antibody libraries.

29. Monoclonal antibodies elicited by a compound with the general formula (1) in claim 1.

30. A method in which a compound with the general formula (1) in claim 1 is used to isolated mutants of natural molecules with improved nucleophilic activity.

31. The method of claim 30 in which molecular arrays of mutants are displayed on the surface of a phage, a virus, a prokaryotic cell or a eukaryotic cell and nucleophilic mutants are isolated by contacting the arrays with a compound of general formula (1) of claim 1.

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APPENDIX

FIG. A

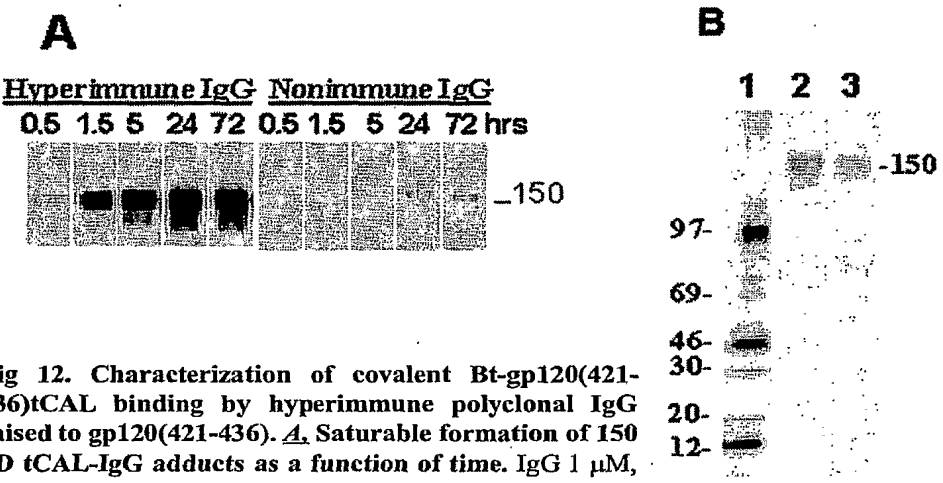
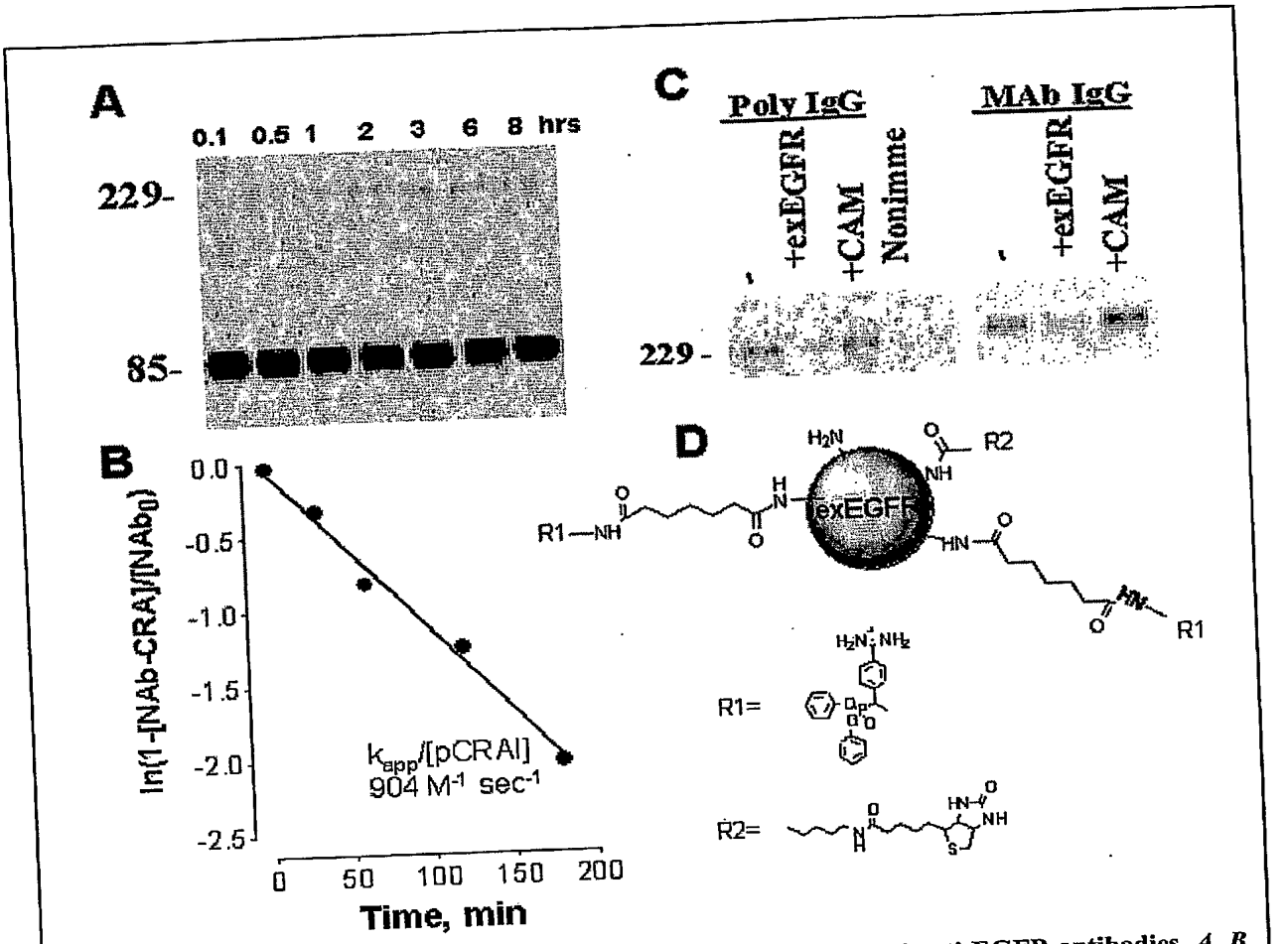


Fig 12. Characterization of covalent Bt-gp120(421-436)tCAL binding by hyperimmune polyclonal IgG raised to gp120(421-436). *A*, Saturable formation of 150 kD tCAL-IgG adducts as a function of time. IgG 1 μM, CAL 10 μM. ***B*, Protection against covalent tCAL labeling by noncovalent binding of gp120(421-436)-BSA (lane 3, 3 μM).** Lane 3, BSA instead of gp120(421-436)-BSA (3 μM). Lane 1, markers. Nonreducing gels, streptavidin-peroxidase stained.

APPENDIX

FIG. B



Functionally coordinated noncovalent and covalent binding activities of anti-EGFR antibodies. ***A, B,*** **Saturable covalent binding of Bt-exEGFR-pCAL as a function of time.** ***C,*** **Protection against pCAL covalent labeling by noncovalent binding of exEGFR.** ***D,*** **Schematic representation of Bt-exEGFR-pCAL labeled at Lys side chains with the biotin and phosphonate diester moieties.** Streptavidin-peroxidase stained nonreducing SDS-gels. Covalent adducts at 229 kD, monomer pCAL at 85 kD. pCAL, 0.2 μM. Polyclonal anti-EGFR, 0.7 μM. Monoclonal anti-EGFR clone c225, 0.5 μM. In C, reaction time was 2 h and the reaction was conducted in the absence (-) or presence of recombinant exEGFR (1 μM) and calmodulin (CAM). No covalent binding detected with nonimmune polyclonal IgG (C) or nonimmune, isotype matched MAb IgG . IgG purified by protein G chromtaography. Polyclonal anti-EGFR raised by immunization of BALB/c mice with exEGFR and A431 cells. kapp/[pCAL] is the second order rate constant.

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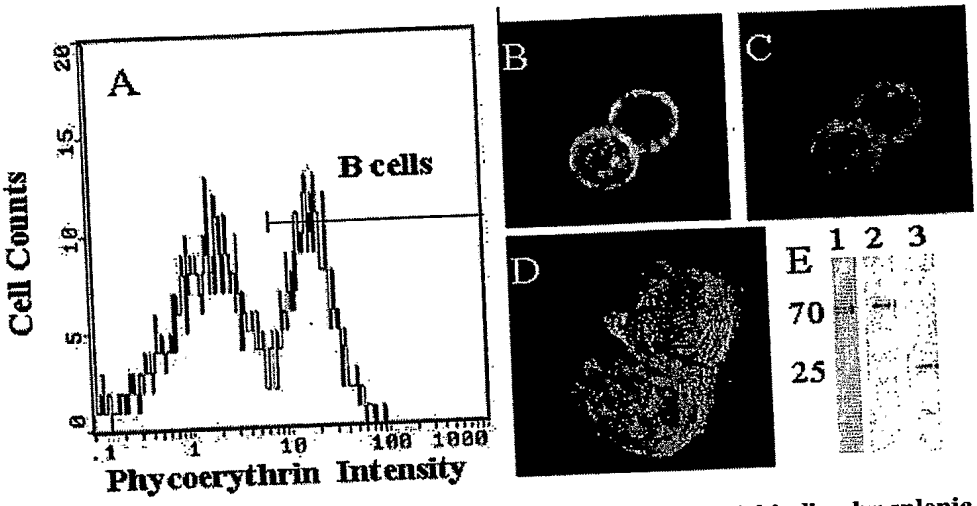
APPENDIX

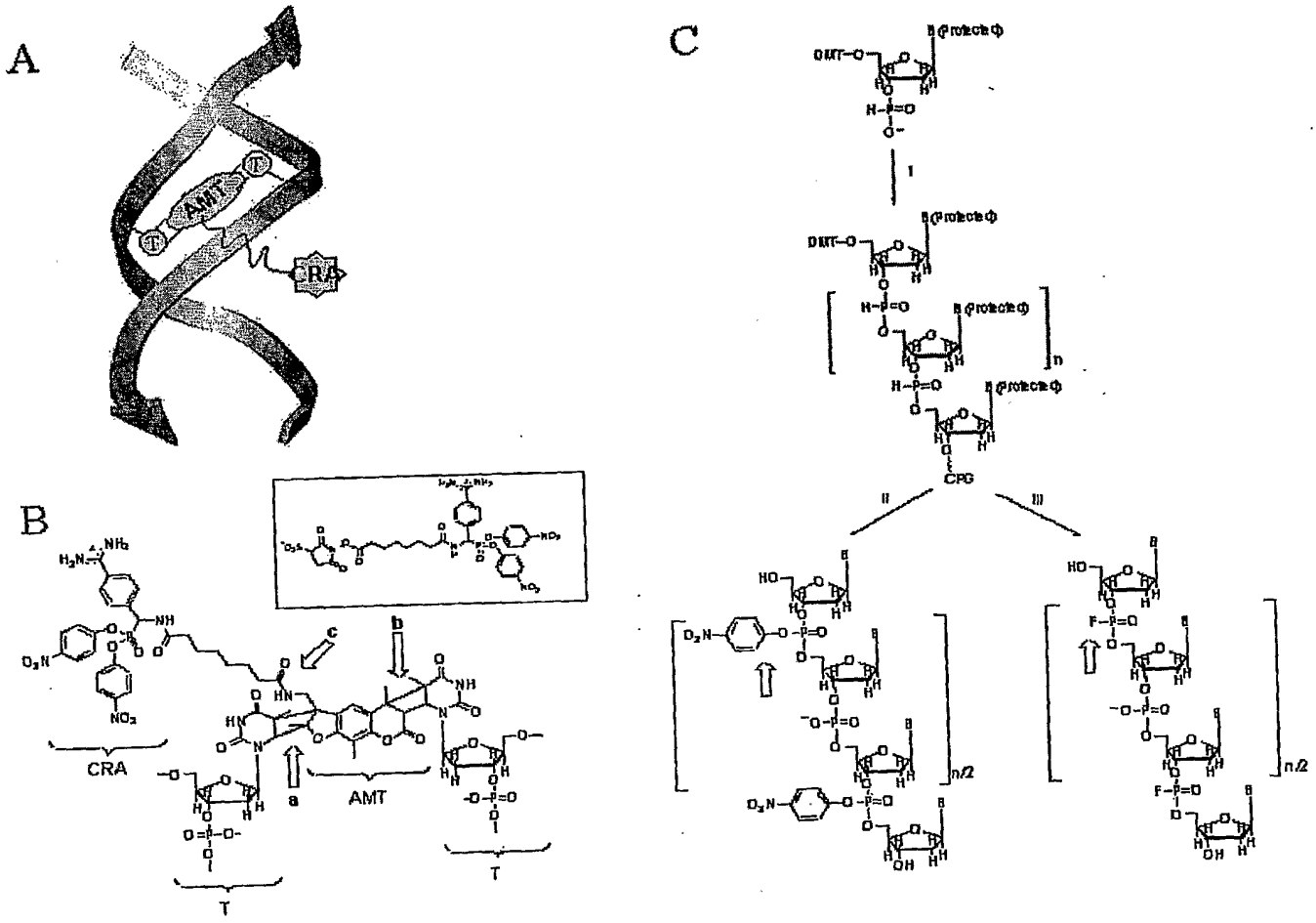
FIG. C



Characteristics of biotinylated diphenyl phosphonate ester CAL binding by splenic B cells (*A-D*) and SDS-gels showing covalent CAL-bound proteins extracted from B cells (*E*). Splenocytes from a BALB/c mouse (5 wks age) were treated with the CAL as in Fig 14 (4 hours), washed with PBS and stained with phycoerythrin conjugated anti-CD19 antibody (1 μg) and FITC conjugated streptavidin (0.1μg). Following PBS washing, the cells were fixed with 2% HCHO, resuspended in PBS and analyzed by flow cytometry. *A* depicts the non-B cell subpopulation (65%) and B cell subpopulation (35%) of the CAL-stainable cell population present in splenocytes (constituting 9 % of total splenocytes). *B* and *C* are confocal micrography of showing two B cells using filters for FITC (bright green) and phycoerythrin (red), respectively (blue counterstain, DAPI). *D* is a 3-d computer model of the emission patterns compiled from 30 individual sections for each emission probe. *E* shows reducing SDS-gels of the detergent extract of CAL-labeled B cells. Lane 1, CAL stained blot visualized with strepavidin-peroxidase. Lane 2, anti-μ chain stained blot. Lane 3, anti-κ/λ chain stained blot. B cells purified by negative selection on magnetic beads (anti-CD43, anti-CD4 and anti-Ter119 Abs).

APPENDIX

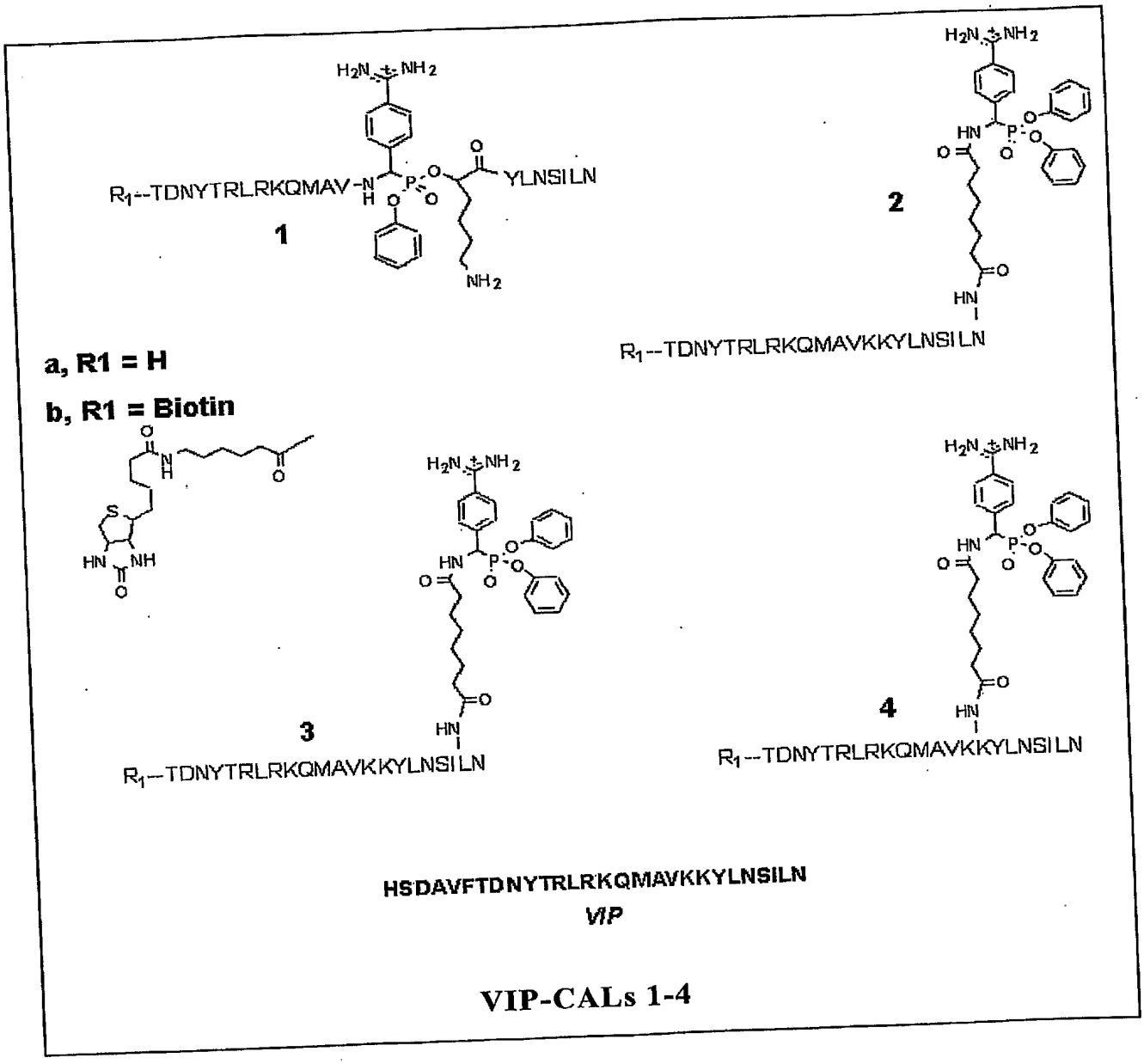
FIG. D



dsDNA-CAL 1 (A,B) and Oligo-CAL 2 (C)

APPENDIX

FIG. E



VIP-CALs 1-4

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Pathogenic Antibodies to DNA and VIP

This example focuses on two targets for developing new treatments for lupus: *(a)* Antibodies (Abs) to DNA; and *(b)* Abs to the neuropeptide vasoactive intestinal polypeptide (VIP). Considerable evidence exists that autoimmune responses to DNA play an important role in pathogenesis of lupus. Ab responses to VIP are a more recently defined target, but their serious consideration is warranted in view of the profound ability of VIP to regulate T cell afferent immune responses and inhibit autoimmune reactions. A majority of Abs display nucleophilic covalent reactivity with phosphonate esters. It appears feasible to recruit classical noncovalent binding forces to deliver covalent inhibitors to Ab nucleophiles, thus imparting specificity to the covalent reaction. The inventor believes that Chemically reactive autoantibodies to DNA and VIP play important roles in the pathogenesis of lupus. The chemical reactivity of the autoantibodies derives from the presence of intramolecularly-activated nucleophilic amino acids. In some instances, the nucleophilicity allows emergence of catalytic activity, which confers increased pathogenicity to the Abs. Probes that bind covalently to DNA and VIP Abs have been developed. The biological consequences of permanentl Ab inhibition by the covalently reactive ligand analogs (CALs) can be studied in culture and in vivo using the MRL/lpr mouse model of lupus.

BACKGROUND

1. Ab nucleophilicity. Protein nucleophilic reactivities derive from activation of the side chains of certain amino acids (Fig 1). In serine proteases, for instance, precise spatial positioning of the Ser-His-Asp triad allows formation of a hydrogen bonded network that imparts nucleophilic reactivity to the Ser oxygen. Until now, nucleophilic reactivity was assumed to be a unique characteristic of enzymes that form covalent reaction intermediates in the course of catalyzing chemical reactions, e.g., certain proteases, glycosidases, lipases and synthases. We observed recently that a majority of Abs express nucleophilic reactivity (including non-enzymatic Abs). The reactivity was identified using phosphonate diester probes, which are well established inhibitors of serine proteases and catalytic Abs (1,2). The P atom in these compounds is electrophilic, and depending on the strength of the leaving group at the ester bonds, the phosphonates form stable covalent complexes with chemically activated nucleophiles. In preliminary studies, the majority of Abs studied formed covalent complexes with biotinylated phosphonates that were stable to denaturing conditions. On a molar basis, the level of Ab nucleophilic activity for haptenic phosphonate esters is comparable to that of trypsin. As the latter protein is highly evolved to maximize its covalent catalytic power, the observed Ab nucleophilicity is unlikely to be a trivial phenomenon. Mechanistic considerations help differentiate between nucleophilic and catalytic proteins, even though both employ similar covalent mechanisms. The covalent reactivity is a necessary but not sufficient condition for catalysis. For example, catalytic cleavage of peptide bonds by serine proteases also requires facilitation of events occurring after formation of the covalent acyl-enzyme intermediate, that is, hydrolysis of the intermediate and release of product peptides (Fig 2). A small subset of nucleophilic proteins is predicted to be catalytic. Thus, the covalent reactivity of noncatalytic Abs, although unexpected, does not present theoretical contradictions with established chemical concepts.

Nucleophilic Abs combine specific recognition of the antigen ground state with covalent phosphonate binding, lending specificity to the reaction. This has emerged from study of nucleophilic Abs with catalytic activity as well as direct analysis of covalent binding of phosphonate-containing antigens. A split site model has been proposed to explain the functional coordination between the covalent and noncovalent reactions (Fig 1). Coordinated nucleophilic and noncovalent epitope-paratope binding has been observed in all Abs examined thus far. Mutating the nucleophilic residue of an Ab did not interfere with initial antigen ground state recognition (3). Moreover, the site of peptide bond cleavage by this Ab is distant from the noncovalently recognized epitope (4). This lead to the hypothesis that distinct Ab subsites are responsible for recognition of the antigen ground state and the chemical reaction. In this model, the nucleophile makes little or no contact with the antigen until the after noncovalent binding is complete. Once initial binding has occurred, flexibility in the active site allows the nucleophile to contact the antigen and initiate the chemical reaction.

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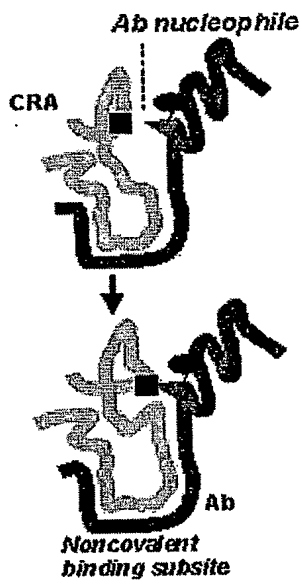

Fig 1. Two step model of CAL recognition by Ab subsites responsible for noncovalent and nucleophilic binding. Initial noncovalent binding does not involve the Ab nucleophile. The residue shown as a triangle gains nucleophilicity by intramolecular interactions The nucleophile forms covalent ligand complex at the phosphorus (rectangle) distant from the noncovalent site.

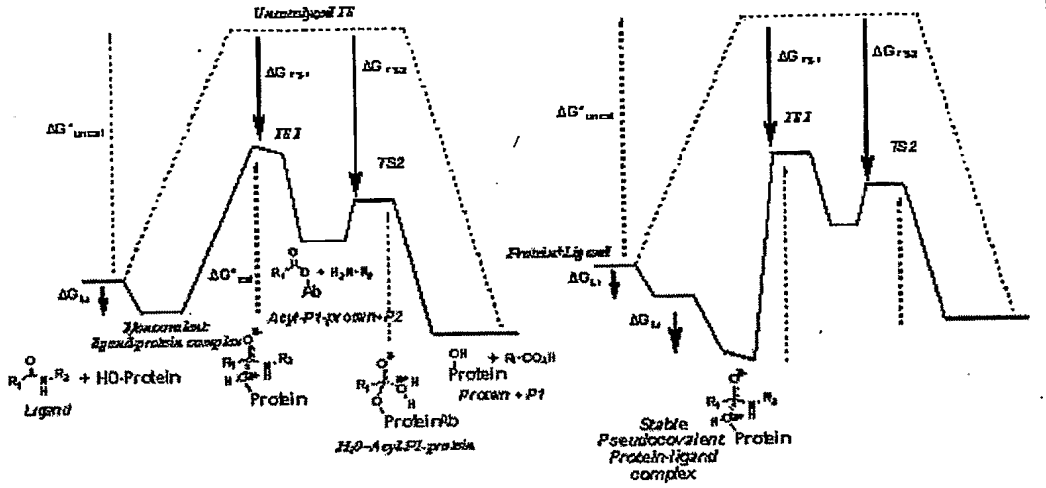

Fig 2: Reaction coordinates for nucleophilic proteins. ***Left,*** Serine proteases stabilize the antigen ground state noncovalently (ΔG_{Ag}). Nucleophiles such as an activated Ser residue attacks the peptide bond, forming an unstable resonant transition state (TS1). Completion of this reaction forms the covalent acyl-Ab intermediate, with release of the C terminal peptide fragment. In the second reaction, a water molecule hydrolyzes the covalent intermediate via a second tetrahedral transition state (TS2). ***Right,*** The reaction proceeds as above, except that the resonant antigen-Ab complex containing partial covalent bonds is more stable than the ground state of the antigen-Ab complex. $\Delta G^{\dagger}_{uncat}$ and $\Delta G^{\dagger}_{cat}$ correspond to activation energies for the uncatalyzed and catalyzed reactions, respectively. K_m is a function of the extent of ground state stabilization (ΔG_S). k_{cat}/K_m is a function of extent of transition state stabilization relative to the catalyst-substrate ground state complex.

2. Nucleophilic antibodies in autoimmune disease. Nucleophilic reactivity enables certain autoantibodies to catalyze the hydrolysis of VIP (5,6). Other reports have indicated a general propensity towards catalytic antibody formation in autoimmune disease (7-13). As noted above, it is important to distinguish between nucleophilic and catalytic Abs, even though both employ similar covalent mechanisms. Nucleophilic Abs can form weak covalent bonds in which resonant structures such as shown in Fig 2, right panel, may stabilize the antigen ground state [a covalent bond is one in which electrons are shared in a common orbital; although such bonds can be stronger than noncovalent bonds, the strength of the bond is not a defining feature of covalency – e.g., a hydrogen bond has partial covalent character]. Nucleophilic Abs may also form stable, dead-end covalent complexes with the antigen - two such examples are published (14,15), and in unpublished studies, we observed stable binding of two antigens (albumin, gp120) by certain Abs that was resistant to SDS treatment. This type of bonding could potentially be involved in formation of certain stable protein-protein aggregates, such as amyloid fibrils of Ab light chains. The aggregates are often stable to denaturing conditions, but no systematic basis for understanding their stability based on noncovalent binding forces is available.

As to immunological genesis, the germline origin of nucleophilic Abs is suggested by observations that IgG Abs in unimmunized humans and animals bind phosphonate haptens covalently and promiscuously cleave model peptide substrates (16). Similarly, covalent labeling of B cell surface Ig by haptenic phosphonates was observed by flow cytometry. The catalytic triad of a VIPase L chain was shown to be present in it germline VL domain counterpart (17), and reversion of the mature L chain to the germline configuration by mutagenesis at 4 residues remote from the triad did not influence the catalytic activity, confirming it's innate origin (18). If promiscuous nucleophile encoded by germline V genes remain in spatial register with the noncovalent binding site over the course of B cell clonal maturation, the nucleophilic reactivity may acquire specificity for individual antigens. This has been verified experimentally (Preliminary Studies). With respect to Ab catalytic activity, chemical transformation of the antigen by the catalysts may lead to its rapid dissociation from the B cell receptor Ig (BCR), which could disfavor routine synthesis of catalysts. The caveat is that slow proteolysis will not influence the antigen-dependent phase of B cell selection as long as the rate of catalysis is lower than the rate of transmembrane signaling needed to stimulate B cell division. Catalytic Abs isolated from subjects with autoimmune disease display high affinities for their autoantigens (e.g., 4,7) and express replacement mutations clustered in the complementarity determining regions, which indicates that the Abs have been subjected to V gene affinity maturation (19). A broad range of polypeptide and nucleic antigens are shown to be susceptible to cleavage by autoantibodies (13). Possible explanations for amplified catalytic Ab production in autoimmune disease are: selective expression of nucleophile-encoding germline V genes, defective somatic diversification mechanisms and disturbed idiotypic network regulation (20,21; reviewed in 22).

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3. Lupus anti-DNA autoantibodies. Anti-DNA Abs are promising candidate targets for lupus therapy. Abs to double stranded DNA (dsDNA) are a diagnostic criterion for lupus, and Abs capable of binding single stranded DNA (ssDNA) and various nucleic acid-protein complexes are found as well (23,24). Abs eluted from the kidneys of lupus patients display strong reactivity with DNA, anti-DNA Abs isolated from the blood of lupus patients bind kidney sections, and adoptive transfer of anti-DNA Abs induces kidney damage in experimental animals (25,26). A major mechanism underlying anti-DNA pathogenicity is thought to entail the following events: binding to DNA-containing nucleosomes or a cross-reactive protein antigen, penetration of the cell and nuclear membranes, and induction of apoptotic cell death (26-29). Some reports suggest that the DNase activity of certain lupus Abs confers enhanced cytotoxic activity (7,30). Another mechanism invokes the deposition of immune complexes by binding to Fc receptors, followed by damage resulting from complement activation. Immune complexes containing DNA of varying size may not be equivalently pathogenic. These mechanisms are not mutually exclusive – for example, anti-DNA Ab binding to nucleosomes may induce complement activation and Ab internalization by the cells may occur as well.

The antigenic specificity of anti-DNA Abs is of considerable importance in designing inhibitors of the Abs. Anti-dsDNA are established to be capable of causing kidney damage, and some evidence is available that anti-ssDNA Abs are also pathogenic (31,32). Abs to dsDNA usually also bind ssDNA, but anti-ssDNA Abs do not recognize dsDNA (23,31-34). The binding site of anti-dsDNA is usually a cleft, whereas anti-ssDNA Abs appear to contain a more shallow site with limited penetration of the DNA into the protein (32,35). Five-mer oligonucleotides are sufficient to bind the Abs, and there is limited specificity for recognition of individual DNA sequences (23,36). However, base preferences are evident, with several anti-dsDNA Abs displaying preference for G-C rich regions, and others, for A-T rich regions regions (23,31,31a). Contacts of Ab combining site residues with negatively charged phosphate groups as well as the bases are described, and in some Abs, base stacking within the combining site allows stabilization of the Ab-DNA complex.

Several studies dealing with pathogenic anti-DNA Abs have relied on the MRL/lpr mouse strain. These mice spontaneously develop lupus-like disease. The disease in MRL/lpr mice is a consequence of a defect in the Fas receptor gene. The resultant apoptotic defect allows unregulated lymphoproliferation, increased production of autoantibodies similar to those produced in human lupus, membranous nephritis, and eventually, death. A monogenic defect is responsible for lupus in MRL/lpr mice and disease progression eventually causes death. The etiology of human lupus is polyfactorial, including various environmental factors and multiple genes, and flare-remission cycles are evident. Similarities in end organ damage in the mice and human lupus are evident, however, and the murine and human anti-DNA autoantibodies display shared specificities.

Targeting Ab binding sites with non-covalent DNA analogs (including small analogs) is limited by the fact that very high affinity analogs must be employed to out-compete excess endogenous DNA. Moreover, reversible binding to the Abs will eventually result in dissociation of the DNA analog, regenerating active Abs. Covalent binding DNA analogs, on the other hand, are intended to permanently engage the Ab active sites. Yet another incentive for pursuing covalent Ab targeting is the probability that covalent occupancy of the BCR will drive B cells into the apoptoic pathway (as opposed to the proliferative pathway induced by noncovalent antigen binding). Permanent (covalent) BCR engagement is analogous to saturation with excess antigen, which is well known to induce B cell clonal silencing (22,37,37a). Thus, inhibition of Ab synthesis by covalent DNA analogs is a feasible outcome in the present project.

4. Lupus anti-VIP autoantibodies. VIP is a 28 amino acid peptide synthesized by neurons and also produced endogenously by T cells. Originally known for the ability to relax the smooth muscle (38), VIP is now recognized as an important regulator of T cells. Although nucleophilic VIP Abs have only recently been recognized as potential targets for lupus therapy, their further study is warranted for the following reasons: ***(a)*** Administration of exogenous VIP completely suppresses rheumatoid arthritis in a collagen-induced animal model of the disease (39); ***(b)*** VIP binding and VIP cleaving autoantibodies are found in patients with lupus, as also in lupus MRL/lpr mice; and ***(c)*** VIP regulates the production of cytokines by T cells (40,41) and aberrant VIP receptor expression on T cells is shown to alter their responses to antigens (42); ***(d)*** Alterations in the tissue concentrations of VIP have been noted in murine lupus (43). Thus, we hypothesize that lupus anti-VIP Abs deplete VIP available to T lymphocytes, resulting in dysfunctional T cell responsiveness to autoantigens. Study of amelioration of murine lupus by covalent VIP analogs will serve as a test for this hypothesis. The advantages of covalent anti-VIP inhibition are essentially as described for anti-DNA Abs, with the important difference that the covalent VIP analogs may correct the afferent limb of dysfunctional T cell responses. Receptors for VIP on lymphocytes must be spared from binding to covalent analogs. Designing peptide analogs that are not recognized by the receptors should not be a problem, as receptor binding requires the entire sequence of VIP, whereas the Abs found in disease recognize mainly the central and C-terminal regions of the peptide (4, unpublished studies).

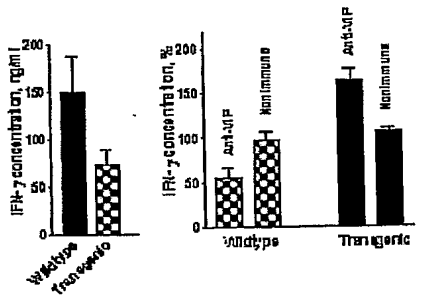

Fig 3: Right, Effect of VIPase IgG on IFN-γ secretion by wildtype and VPACR2 transgenic CD4+ cells (splenocytes). Left, Comparison of IFN-γ secretion by the wildtype and VPACR2 transgenic cells. c23.5 VIPase and isotype-matched nonimmune IgG, 7 nM; 96 h incubation with CD4+ cells ($3x10^5$/well) prepared using immobilized anti-CD4 Ab. IFN-γ measured by ELISA. Data are means ± s.d. (N=3).

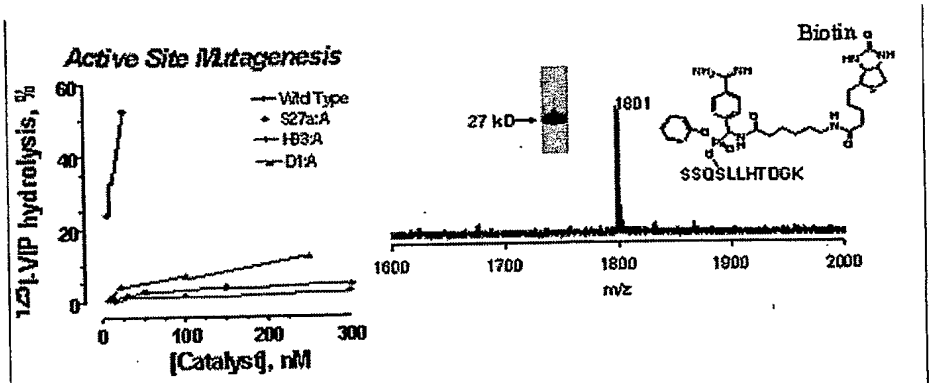

Fig 4: VIPase Ab c23.5 characteristics. ***Left,*** **Hydrolysis of VIP by the recombinant L-chain and its catalytic triad mutants.** 100 pM ^{125}I-VIP; 3h. ***Right,*** **Mass spectrum (MALDI-TOF) of CAL labeled L chain nucleophilic fragment.** Purified L chain (100 μg) labeled with CAL (100 μM), reduced and alkylated (MeSH, iodoactemide), digested with trypsin (30 μg, 2 h), biotinylated peptide fragments purified on an anti-biotin column (elution in 8 M urea). MALDI in α-cyano-4-hydroxy cinnamic acid matrix. The major 1801 amu peak represents monophenyl phosphonylated peptide residues 25-35 containing the Ser 27a nucleophile. **Inset,** streptavidin-peroxidase stained SDS-gel showing the CAL-L chain complex.

EXAMPLES SUPPORTING THE INVENTION: (please see Appendix for certain figures cited herein).

1. Autoimmune anti-VIP Abs: Details of human autoantibody VIPases have been reported (4,6,19,45-48), i.e., affinity, turnover, identity of cleavage sites, epitope specificity, and presence of activity in Fab fragments and the light chain subunit. Briefly, VIP cleaving Abs are present in the serum of patients with lupus, autoimmune thyroiditis (ATh) and asthma, but not in healthy control subjects (Appendix, Fig 1 in Publ 3). Serum VIPase Abs are also present in lpr and gld mouse strains, which develop lupus-like disease. VIPases are detected in the lpr strain as early as age 8 wks. The activity is VIP-selective, indicated by lack of cleavage of irrelevant polypeptides (6,19). Conventional ELISA indicated increased VIP binding Abs in lupus patients and lupus mice (lpr and gld strains), but the disease-association of the binding activity is weaker compared to VIPase activity (Appendix, Publ 4). Certain recombinant Fv clones and light chains isolated from human autoimmune phage display libraries (lupus; asthma) displayed saturable VIPase activity, and others Fv clones displayed specific VIP binding, confirming that the active site is located in the V domains (1,19; Appendix Publ 3,4). The overall catalytic proficiencies $[(k_{cat}/K_m)/k_{uncat}]$ are better than that of trypsin. Several VIPase Fv and L chains containing highly mutated V domains have been identified, suggesting that autoantibody catalytic activity is compatible with V region affinity maturation.

Potent effects of catalytic Abs are anticipated because of permanent target inactivation and turnover of multiple target molecules by a single catalyst molecule. Direct evidence for increased potency due to the catalytic function was obtained from study of VIP binding to tissue receptors following incubation with the wild type and His93:Arg mutant of the c23.5 L chain (49). The mutant and wildtype L chains bind VIP with equivalent affinity, but the mutant is 100-fold less active catalytically (50). Near-complete inhibition of VIP binding to the receptors by the wild type L chain was evident but the mutant effect was only marginal. Previously, we showed polyclonal anti-VIP Abs to suppress VIP-induced synthesis cAMP, the second messenger utilized by VIP (47). In collaboration with Dr. E. Goetzl (UCSF), VIPase IgG c23.5 was observed to induce profound changes in cytokine production by T lymphocytes (51). T cells from transgenic mice overexpressing VIP receptor subtype 2 (VPACR2; this receptor is associated with T cell activation) were employed. These cells express altered cytokine secretion pattern compared to wildtype lymphocytes (52). Treatment of the lymphocyte cultures with the VIPase IgG corrected the deviation in IFN-γ (Fig 3), IL-4 and IL-10 (not shown) secretion from the VPACR2 overexpressing cells. Total VIP levels in the VIPase-containing cultures were reduced by >80%. In a study dealing with pulmonary immunological regulation by VIP, administration of VIPase IgG or the isotype-matched nonimmune IgG to BALB/c mice resulted in a statistically significant increase ($P<0.01$) increase in the number of CD4+ T lymphocytes in the bronchoalveolar lavage fluid determined by flow cytometry. Evidence for the ability of the VIPases to influence the biological effects of VIP in vivo is also available from study of pulmonary smooth muscle function (49).

2. Nucleophilic mechanism. Fv and L chain fragments of an anti-VIP monoclonal Ab were cloned in a bacterial expression (53). The purified 27 kD L chain and Fv constructs expressed VIPase activity, with the cleavage reaction occurring predominantly at K21-Y22 and K20-K21 bonds (54). Molecular modeling indicated that VL residues Asp1, Ser27a and His93 are positioned as a catalytic triad similar to that in serine proteases. Ala-replacement mutations at these residues reduced the hydrolysis of VIP

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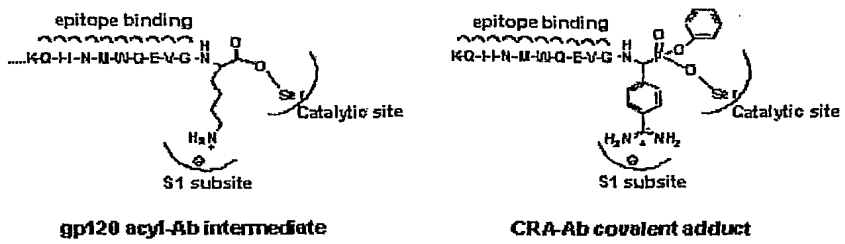
by >90% (Fig 4) (49). The catalytic activity was inhibited by diisopropylfluorophosphate (DFP), a covalent serine protease inhibitor. The nucleophilic reactivity was evident from observations of covalent adducts of the L chain and Fv with biotinylated phosphonate diesters (see 67 for phosphonate synthesis). Tryptic digestion of the reduced and alkylated adducts allowed mass spectroscopic identification of a phosphonylated CDR1 peptide corresponding to residues 24-32, consistent with assignment of Ser27a as the nucleophile (Fig 4). Inhibition of the proteolytic activity by DFP and hapten CALs has been observed consistently for other Abs that catalyze the cleavage of VIP (1), thyroglobulin (9), gp120 (55,56) and model peptide substrates (16). The inhibition is irreversible and excess antigen protects against inhibition, indicating that DFP binds at the Ab active site (57). Several biotinylated haptenic CALs with leaving groups of different strengths were employed to assess the nucleophilic reactivity of polyclonal IgG from the serum of healthy humans and nonimmune mice. As the CALs are devoid of peptidic epitopes, they bind Abs without the influence of noncovalent paratope-epitope interactions. No IgG adducts of methyl containing CALs (which is a weak leaving group) or of neutral CALs devoid of the amidino group were detected. In contrast, the phenyl and amidino-containing CAL shown in Fig 4 formed adducts stable to denaturing electrophoresis conditions with each IgG samples from 5 healthy humans and pooled IgG from unimmunized BALB/c mice. CAL-labeled H chain subunits were evident in addition to the labeled L chains by reducing gel electrophoresis. Although purified H chains devoid of L chains have not been shown to possess catalytic activity, two previous studies have implicated Ser nucleophiles located in the H chain as catalytic residues responsible for IgG catalyzed ester hydrolysis (58,59). Adduct formation was inhibitable by DFP and no adducts were formed by heat-denatured IgG, indicating the importance of the native IgG conformation. All six randomly picked Fv clones from a human Fv library formed detectable hapten CAL adducts, indicating that the nucleophile(s) is located in the V domains. Eight Fv and 11 L chains from the phage display libraries were analyzed for CAL binding and catalytic cleavage of Pro-Phe-Arg-MCA (as in 1). Values of correlation coefficients (r^2) were 0.75 ($P<0.005$) and 0.32 ($P<0.05$) for the Fv and L chains clones, respectively. We concluded that CAL binding serves as a predictor of catalytic activity. Covalent trapping was employed to select catalysts from phage displayed Ab repertoires (1). The biotin tag allows capture of CAL-bound Ab phages using immobilized streptavidin. Reduction of the S-S bond between the phosphonate diester and biotin allows recovery of covalently bound phage particles. Selected L chains and Fv constructs were expressed in soluble form in bacteria and purified by metal affinity chromatography. The selected Abs displayed enriched catalytic activity for model peptidase substrates containing a basic residue at the cleavage site. Turnover values (k_{cat}) for a selected Fv and a L chain against their preferred model peptide substrates were, respectively, 0.5 min^{-1} and 0.2 min^{-1}, and corresponding Michaelis-Menten constants (Km) were, 10 μM and 8 μM.

3. Germline origin of nucleophilic reactivity: The catalytic triad of VIPase L chain c23.5 is present in its germline VL gene counterpart (GenBank # Z72384), but the mature form of the L chain contains 4 replacements (His27d:Asp, Thr28e:Ser, Ile34:Asn and Gln96:Trp; germline residues shown second). The germline counterpart of the anti-VIP L chain was constructed by introducing the required 4 mutations. The purified germline protein expressed expressed catalytic activity as detected by cleavage of VIP and Pro-Phe-Arg-MCA substrate (18), indicating that the remote somatic replacements are important for the integrity of the catalytic triad. Haptenic CAL binding studies of the germline L chain as in (1) confirmed the expression of nucleophilic reactivity by this protein.

4. Split-site model of Ab active sites. Initial evidence for this model came from studying the reaction of polyclonal VIPase IgG with synthetic subsequences of VIP (4). The C terminal peptide VIP(22-28) was bound by the Abs and inhibited the hydrolysis of VIP at a remote site competitively ($(Q^{16}\text{-}M^{17})$, suggesting that distinct regions of VIP are recognized in the noncovalent binding and chemical steps. Further support is provided by observations of unaltered K_m ($\sim K_d$) of VIPase L chain c23.5 by mutations at the catalytic residues (Ser27a, His93, Asp1) (49). VL-VH interactions constitute yet another mechanism for remote control of the catalytic site. Linking the VIPase VL with its VH domain partner yielded an Fv with improved VIP binding affinity (K_d 5 nM) and catalytic proficiency (by 7 fold) relative to the L chain (54). In comparison, an Fv composed of the VIPase VL domain linked to an irrelevant VH domain (anti-lysozyme) displayed considerably reduced VIP cleavage.

Message-address systems were devised based on the split site model. Binding at the noncovalent Ab site (address) is exploited to achieve antigen delivery to the Ab nucleophile (message). In one application, this system was applied to Abs to the HIV coat protein gp120 (for details, see Appendix, Publ 1,2). Lupus patients express Abs to a conserved peptide determinant of gp120, residues 421-436. gp120(421-431) with a phosphonate diester in place of Lys432 at the C terminus and biotin at the N terminus was synthesized (tCAL; Fig 5). Catalysts were selected by covalent tCAL binding of lupus Fv and L chain phages (1). Two gp120-cleaving catalysts were identified by screening the selected Fv and L chain clones. One L chain has been characterized



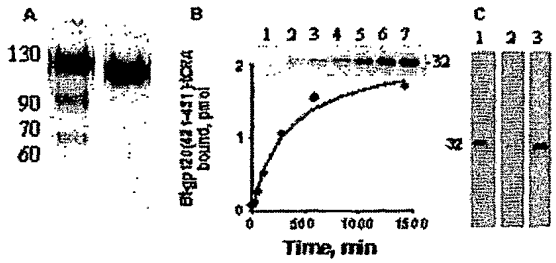


Fig 5. ***Top.*** **Schematic diagrams showing design elements underlying peptidyl CAL (tCAL) mimicry of Ab interactions with the gp120(421-431).** ***Bottom A,*** **SDS-electrophoresis gels showing gp120 cleavage by light chain clone SKL6 (left lane) and control gp120 treated with a noncatalytic L chain (right lane).** gp120 (SF2) biotinylated at Lys residues (0.3 mol biotin/mol gp120; 0.03 μM); SKL6 0.03 μM, 21 hours incubation. Blot stained with streptavidin-peroxidase. ***B.*** **Covalent L chain binding of Bt-gp120(421-431)tCAL as a function of time.** L chain 1 μM, CAL 10 μM. Inset, streptavidin-peroxidase stained blots of SDS-gels corresponding to the time points in the graph. ***C.*** **Active site protection by gp120(421-436).** Shown are SDS gels of covalent Bt-gp120(421-436)tCAL (10 μM) adducts formed with the L chain (1 μM) in the absence (lane 1) and presence of gp120(421-436) (10 μM; lane 2) and an irrelevant peptide [EGFR(351-364), 10 μM; lane 3].

further (clone SKL6). This L chain contains 3 replacement mutations compared to its germline V gene counterpart O2/O12 (Arg18:Gly,Asn34:His, Ala50:Val). Covalent tCAL binding by the L chain was inhibited by gp120(421-436) but not by equivalent concentrations of an irrelevant peptide (synthetic EGFR peptide 294-310), indicating active site protection by the noncovalently bound antigen. Time-dependent accumulation of tCAL-L chain adducts was evident. The second order rate constant kapp/[tCAL] value deduced from the saturation curve was 1.60 M^{-1} sec^{-1} compared to a value of 0.06 M^{-1} sec^{-1} for trypsin, indicating superior nucleophilic reactivity of the L chain. Cleavage of gp120 by the L chain occurs with a submicromolar Km value. No cleavage of biotinylated BSA or biotinylated extracellular domain of EGFR (exEGFR) was evident (not shown).

Two examples of phosphonate delivery to noncatalytic Abs are described below. First, polyclonal IgG raised by immunization with gp120(421-436) was analyzed for covalent binding to the peptidyl gp120(421-431)tCAL (Appendix, Fig A). Electrophoresis of IgG treated with the tCAL indicated saturable accumulation of the 153 kD covalent adducts (IgG, 150 kD; tCAL, 2.2 kD). The second order rate constant (kapp/[tCAL]) computed from the saturation was plot is 18.4 M^{-1} sec^{-1}, compared to a value of 0.1 M^{-1} sec^{-1} for trypsin. Covalent tCAL binding by nonimmune IgG was virtually undetectable. Formation of adducts was inhibited by treatment of IgG with gp120(421-436)-BSA but not BSA, indicating that the covalent reaction must occur close to the noncovalent peptide binding site. In the second example of facilitated phosphonate delivery, the phosphonate diester groups were placed on Lys side chains of a large protein [the extracellular domain of EGFR (exEGFR)]. SDS electrophoresis of biotinylated exEGFR-pCAL (3 mol biotin and 15 mol CAL/mol exEGFR) yielded a single silver stained band at 85 kD. Polyclonal IgG raised to EGFR (60) formed covalent complexes with Bt-exEGFR-pCAL, evident from saturable accumulation of a 235 kD band (Appendix, Fig B; IgG, 150 kD; exEGFR-pCAL, 85 kD). The 235 kD adduct band was stainable by an anti-mouse IgG-peroxidase conjugate in immunoblots (not shown). The overall covalent binding efficiency assessed from the second order rate constant was 904 M^{-1} sec^{-1}. The impressive nucleophilic reactivity presumably derives from noncovalent delivery of the protein to the nucleophile, as covalent binding to nonimmune IgG was not detected. All 3 commercially available anti-exEGFR monoclonal Abs (clones C225, H11 and C111.6; Labvision, CA) formed covalent Bt-exEGFR-pCAL adducts. No adducts were formed by a control monoclonal Ab to BSA. The covalent delivery system works despite unpredictable spatial positioning of the phosphonate. Its success may be explained by the flexibility enjoyed by the antigen-Ab complex, which allows alignment of the two apposing molecules to approach optimal levels by movement of the peptide backbone and amino acid side chains.

5. DNase Abs. Dr. S. Rodkey (coinvestigator) has previously reported the dsDNA cleaving activity of a single chain Fv cloned from an anti-DNA Ab (61). ssDNA is also utilized as a substrate, including short 8-mer oligonucleotides. Similar results are reported for polyclonal IgG fractions isolated from lupus patients (62). We screened 30 recombinant L chains from our lupus library purified as in (1) for dsDNA cleaving activity using plasmid pASK40 as the substrate (Fig 6). Ten L chains displayed detectable DNA-nicking activity, with one clone showing activity at concentrations as low as 0.8 nM (KM38). As the L chains were purified by the same method (metal affinity chromatography from recombinant bacteria grown in parallel), it is difficult to explain these results as being due to DNase contamination. Treatment of a kidney cell line with L chain KM38 resulted in cell death, measured by the MTT assay, as reported previously for L chains isolated

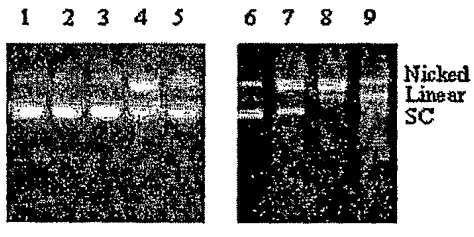


Fig 6. DNase activity of human lupus L chain clone KM38 (lane 4, 50 nM) and MRL/lpr IgG (lanes 7,8,9 -- 20, 100, 500 nM, respectively). Substrate, pASK40 dsDNA 5μg; 3h. 1% agarose gel. SC, supercoiled.

from multiple myeloma patients (63). In comparison, an L chain devoid of DNA cleaving activity did not exert a cytotoxic effect. Related studies have indicated that DNase L chains capable of cellular internalization tend to be cytotoxic (63). Similar findings are published with respect to polyclonal IgG from lupus patients (30). IgG isolated from MRL/lpr mice displayed DNA cleaving activity. Remarkably, the activity in IgG from mice that were 8 weeks old was 9.2-fold greater than IgG from 20 week old mice (N=5 each; P<0.01). DNA binding Abs on the other hand, are known to increase as the mice age. Immunoadsorption and Fab fragment studies of IgG have suggested that contamination with blood-borne DNase is not a factor.

6. CAL binding by splenocytes. Study of hapten CAL binding by splenocytes from unimmunized mice have identified Ig heavy and light chains as the major protein nucleophiles on the surface of B cells. Splenocytes were treated with the biotinylated hapten CAL (see Fig 4 for structure) and bound CAL was visualized using streptavidin-FITC (Appendix, Fig C). An intensely stained subpopulation of the cells was evident in three repeat experiments (~12% of total cells). No loss of viability was evident during CAL incubation determined by trypan blue exclusion (90 and 91% viability before and after incubation for 30 min). The phosphonic acid analog of the CAL, which is devoid of covalent reactivity with protein nucleophiles, did not stain the cells. Flow cytometry using phycoerythrin conjugated anti-CD19 indicated that 35% of the stained cells were B cells. Confocal microscopy of B cells double positive for CAL staining and anti-CD19 staining suggested that the bound CAL was located on the cell surface. Extraction of purified B cells with the detergent CHAPS (9 mM) followed by reducing SDS-electrophoresis yielded 2 major biotin-containing bands at Mr 70 and 26 kD that were immunoblottable with Abs to μ chains and κ/λ chains, respectively.

7. CALs. Concepts derived from study of nucleophilic anti-peptide Abs are applicable to anti-DNA Abs for the following reasons: formation of protein-DNA covalent intermediates by nucleophilic centers in certain DNA cleaving enzymes is a well known phenomenon; hapten phosphonate probes identify Ab nucleophilic reactivity independent of paratope specificity; and, the Ab nucleophilic activity is germ-line encoded and there is no reason to expect that the activity will be lost as anti-DNA Abs undergo affinity maturation. DNA-CAL and VIP-CAL design is based on the split site model, in which the Ab nucleophile is spatially separated from the non-covalent antigen binding site. In the reaction scheme of Fig 7, CALs that fully mimic reversible antigen binding to Ab will possess low K_i (equilibrium dissociation constant) and large k_3 (strength of covalent reaction). These are desired CAL kinetic properties.

$$\text{Ab} + \text{CAL} \underset{}{\overset{K_i}{\rightleftharpoons}} \text{Ab-CAL} \xrightarrow{k_3} \text{Ab-CAL}$$

Fig 7. Reaction scheme for initial noncovalent Ab-CAL binding and the subsequent covalent binding reaction. Hydrolysis of the Ab-CAL complex is assumed to be negligible..

8. DNA-CALs (see Appendix, Fig D for structures). Several types of CALs can be readily prepared, e.g., the large dsDNA-CAL 1 and a single stranded oligonucleotide-CAL 2 (oligo-CAL 2). The starting material can be calf thymus dsDNA digested with restriction enzymes to yield ~200 bp fragments. Use of heterogenous dsDNA is desirable to target the spectrum of autoantibodies to dsDNA found in lupus. [Note: smaller dsDNA-CALs can be readily prepared based on findings that lupus anti-DNA Abs display preferences for A-T rich structural cores of DNA (e.g., ref 36)]. dsDNA-CAL will be prepared by the photochemical reaction 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT). The primary reaction is cyclobutane ring formation between the 5,6 double bond of thymidine in DNA and the psoralen 4',5' or 3,4 double bonds. Reaction at the 4',5" double bond (arrow **a**) results in a monoadduct, which reacts further with a pyrimidine on the complementary strand (arrow **b**) forming an interstrand cross-link (A and B) (64). The pre-activated biotin-containing CAL moiety (inset) is linked to the amine of AMT (arrow **c**). Reaction stoichiometry will be optimized to yield adducts containing 1 CAL/30 bp. This is intended to enable covalent Ab binding without disrupting overall DNA structure. CAl density can altered if needed after CAL validation data are available (see below). Psoralen incorporation is determined by measuring primary amines. CAL incorporation is determined by photometric estimation of 4-nitrophenol after complete alkaline hydrolysis. In the case of the oligonucleotide, covalent reactivity is introduced by modifying backbone phosphate diester linkages to triesters or fluorophosphate diesters. [Introducing phosphonates into the backbone is not useful, as alkoxy groups withdraw electrons poorly, with the result that the P remains insufficiently electrophilic.] Arrows in the figure indicate the potential leaving groups in the oligo-CAL. The precursor, an H-phosphonate DNA analog (compound **i**), is used as an intermediate in conventional solid-phase DNA synthesis (65). After removing protecting groups with aqueous ammonia, 1 in 5 backbone H-phosphonate functionalities are converted to 4-nitrophenyl phosphonates using the appropriate amount of 4-nitrophenol (compound **ii**). Unreacted H-phosphonates are then oxidized with iodine to give ordinary phosphate diester linkages interspersed with the triester. For fluorophosphate CAL synthesis, the product is treated by customary DNA synthesis methods (I oxidation/aqueous NH3) and then with KF in the presence of 18-crown-6 thionyl chloride (**iii**). This converts the phosphate diester to fluorophosphate diester (KF will be titrated to yield 1 fluorophosphate group/5 bases). Derivitization is estimated by photometric measurement of 4-nitrophenol after alkaline hydrolysis or fluorine quantification by elemental analysis. Oligo-CAL length can be held at 30 bases. Biotin is attached at the 5'terminus of the oligo-CAL during synthesis. Although the CAL moieties are randomly positioned, their density is sufficient to afford Ab binding (minimum epitope length, 5 nucleotides). Lupus anti-ssDNA Abs display preference for poly(dT) stretches (23,32) but their specificity for individual sequences is comparatively unrestricted. Accordingly, the central region of the oligo-CAl can be a 16-mer flanked by 7-mer random sequences on each side [$(N)_7$-$(T)_{16}$-$(N)_7$], with degeneracies in the flanks introduced using mixtures of A/T/G/C during

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synthesis.

9. VIP-CALs (see Appendix, Fig E for structures). VIP-CALs are derived from full-length VIP or from a truncated form of the peptide. A truncated sequence is necessary to avoid receptor binding [the entire VIP sequence is needed for binding by receptors; autoantibodies recognize the C-terminal half of VIP]. A phosponate diester serves as the covalent moiety, and a positively charged amidino group is placed on the N terminal side of the phosphonate, which is a K/R mimetic corresponding to the CAL.binding specificity of nucleophilic Abs. The N-terminal biotin allows detection of Ab binding. VIP-CAL 1 contains the the amidino-phosphonate diester incorporated within the peptide backbone in place of K20. The peptide flanks are desirable to more fully capture noncovalent paratope-epitope interactions. VIP-CAL 2-4 contain the phosphonate located on NH2 side chains of the 3 K residues in VIP. Standard chemical synthesis techniques are applied (66-69, Appendix Publ 1). VIP-CAL 1 is prepared by synthesizing the phosphonate diester unit in solution initially. The protected peptidyl phosphonate is coupled to the C-terminal peptide fragment prepared by solid phase synthesis using an N-terminal glycolate residue, and the product is treated with anhydrous TFA to give VIP-CAL 1. CALs 2-4 will be prepared by conjugating diphenyl amino(4-amidinophenyl) methanephosphonate and VIP (or biotinylated VIP) using the homo-bifunctional linker, bis-sulfosuccinimidyl suberate, followed by separation of individual isomers labeled at at different Lys residues by HPLC. CAL identity is confirmed by mass spectroscopy/NMR. The position of the acylated Lys is determined by MS/MS analysis.

10. Covalent Ab binding. The following steps can be undertaken to validate CAL design: ***(a)*** determine the magnitude and characteristics of covalent CAL binding by lupus Abs; and ***(b)*** determine whether CAL binding induces loss of Ab DNA/VIP binding and DNase/VIPase activities. Separate study of CAL effects on Ab binding and catalytic activities is desirable because the latter activity may be intrinsically more pathogenic. Thus, even if CAL do not fully inhibit the binding activity, a useful effect may be obtained if catalysis is blocked. Polyclonal Abs are employed for most validation studies, as diverse anti-DNA/anti-VIP Abs found in vivo must be targeted. In some situations, use of monoclonal Abs is needed, e.g., to accurately determine kinetic constants. Pooled IgG from the following sources can be studied: ***(a)*** lupus patients diagnosed according to the Am Rheumatol Soc criteria along with control subjects (N=20); ***(b)*** 8 wk old MRL/lpr mice and control MRL/++ mice (N=8); ***(c)*** 20 week old MRL/lpr mice and control MRL/++ mice (N=8). IgG purified on protein G-Sepharose is treated with increasing CAL concentrations. Covalent binding is determined by SDS-electrophoresis and staining of biotinylated bands in blots using streptavidin-peroxidase and a chemiluminescent substrate. In the case of DNA-CALs, mass determination of the complexes is not possible, but unambiguous separation of Ab-complexed and free DNA-CAL will occur. Controls include treatment with excess DFP (competitor for binding at the Ab nucleophile) and excess DNA/VIP (noncovalent competitor; this will show that CAL binding occurs at the active site). The reaction is quantified by densitometry using a Biorad imager (linear over 5 log orders of biotin).

Noncovalent binding at antigenic epitopes is anticipated to allow superior VIP-CAL and DNA-CAL binding by lupus Abs compared to control Abs. To confirm that ambiguities related to the polyclonal nature of the IgG are not a factor, the CALs will be analyzed using at least one monoclonal Ab to DNA and VIP (e.g., clone BV04-01 for the DNA-CAL; clone c23.5 for VIP-CALs). CAL inhibition of Ab catalyzed DNA and VIP cleavage is determined using lupus polyclonal IgG along with available monoclonal DNase and VIPase Abs (e.g., DNase clones BV04-01, KM38; VIPase clones hk14, DM506). Electrophoresis and radioimmunometric methods described by our group previously will be used for this purpose. CAL binding and inhibition of conventional enzymes will be studied similarly to confirm CAL selectivity (serine acylases, e.g., trypsin; DNA cleaving enzymes, e,g, DNase I). Kitz-Wilson plots of covalent binding as a function of time and CAL concentration (67) allows estimation of Ki (strength of noncovalent binding) and k3 (strength of nucleophilic reactivity). Irreversibility is established by protein G chromatography of Ab-CAL complexes (to remove free CALs) followed by reanalysis of DNA and VIP binding. Loss of binding activity indicates covalent Ab inhibition. Standard ELISA methods aree employed for this purpose, in which the antigen (DNA, VIP) is immobilized on the solid phase and bound Abs are determined using peroxidase conjugated anti-human/mouse IgG. ELISA studies are done using streptavidin plates coated with synthetic VIP/oligonucleotide biotinylated at the N terminus and 5' end, respectively. dsDNA and ssDNA from calf thymus are immobilized directly on the plates (ssDNA prepared as in ref 70).

Use of previously employed diphenyl phosphonate diesters in the dsDNA-CAL ensures that the covalent reactivity of this compound is sufficient to probe anti-DNA nucleophilicity. The oligo-CAL contains phosphate triesters/fluorophosphate diesters in its backbone, the covalent reactivity of which should be comparable to diphenyl phosphonates and DFP. VIP-CAL design and synthesis is modeled on our previous studies on polypeptide CALs. As diverse Abs present in lupus are targeted, any one CAL may not be sufficient to obtain complete or near-complete covalent Ab blockade. Thus, it may be necessaru to use mixtures of the CALs in subsequent studies. High affinity binding due to noncovalent paratope-epitope recognition will minimize CAL reactivity with irrelevant Abs and enzymes. However, we recognize that it is necessary to strike a balance between the level of heterogeneity present in the CAL preparation and the desired selectivity, as greater CAL heterogeneity increases the risk of unintended cross-reactions. This issue is relevant for the DNA-CALs, but the problem is minimal with respect to VIP-CALs, as Abs to VIP recognize more well-defined epitopes.

11. DNA-CAL *inhibition of* Ab cytotoxicity. Lupus anti-DNA Abs can traverse membranes and induce apoptosis. Cytotoxic

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effects of anti-DNA Abs treated with DNA-CALs can be assessed using renal tubular cell line (LLC-PK) and an MTT assay (8). The IgG source is lupus patients, healthy human subjects, 8 wk old MRL/lpr mice, 20 week old MRL/lpr mice and control MRL/++ mice at equivalent ages. In addition, at least one monoclonal L chain is analyzed to exclude confounding effects due to Ab heterogeneity (e.g., clone KM38). IgG is incubated in diluent or various concentrations of DNA-CALs and control DNA (devoid of the CAL moiety), free ligand is removed as in Aim1, and the mixtures mixed with cells cultured in 24-well plates. Cytotoxicity is quantified after 24 h. Control wells receive no Ab. Confirmation is obtained by microscopic inspection after staining with propidium iodine (dead cells) and Hoechst 33342 (dead+live cells). Additional studies are done by treating the cells with Abs and CALs without removal of free CALs – these are needed to show that the CALs do not themselves exert a cytotoxic effect, and that a beneficial effect is obtained when the CALs and the cells compete with each other for Ab binding. Direct cytotoxic effects of lupus Abs in the kidney are generally conceived to involve Ab binding to DNA-containing antigens and/or cross-reactive protein antigens, followed by penetration of the cell/nuclear membranes by pathways that are remain to be defined fully. Covalent Ab engagement by CALs should permanently knock out the antigen binding function. Thus, unless an Fc effector function is involved, CAL-bound Abs are anticipated to be devoid of cytotoxic effects (note that there is no source of complement or ADCC-competent cells in the cultures, minimizing the likelihood of Fc involvement).

12. DNA-CAL inhibition of kidney antigen recognition. To the extent that antigen recognition is the mechanism for deposition of lupus Abs in the kidney, covalent blockade of the Abs by DNA-CALs should result in loss of Ab binding to kidney sections. The quantitative importance of antigen recognition versus Fc receptor binding as mechanisms for Ab deposition in the lupus kidney is not known. Kidney cryostat sections from wild type MRL/++ mice as well as MRL/lpr mice are analyzed, as increased nucleosome formation in lupus is suggested to allow increased opportunity for anti-DNA Ab binding (71). Immunofluorescence methods are employed to determine binding of control IgG and IgG treated with DNA-CALs (IgG from 8 wk old MRL/lpr mice, 20 week old MRL/lpr mice and equivalently aged control MRL/++ mice; anti-mouse IgG-FITC conjugate). To determine whether residual binding by CAL-derivitized Abs involves Fc receptors, the binding studies are repeated in the presence of excess mouse Fc fragments (prepared by papain digestion/protein-A chromatography).

13. VIP-CAL effect on cytokine synthesis. VIP exerts important effects at several critical steps in T cell differentiation, including cytokine synthesis and expression of membrane proteins (e.g., Fas ligand expression, costimulatory protein B7.2). The beneficial effect of VIP in suppressing autoimmune responses is known (39). VIP-CALs are studied for the ability to correct changes in T cell cytokine synthesis induced by anti-VIP Abs. T cells from transgenic mice overexpressing the type 2 VIP receptor (VPACR2) are employed as in previous studies (51). T-helper responses are associated with VPACR2 upregulation, which makes this experimental system directly relevant to lupus. Readily detectable changes in cytokine synthesis accompanied by depletion of VIP are observed by treating the VPACR2 overexpressing T cells with an anti-VIP Ab. T cells isolated from the mice using anti-CD4 magnetic beads are cultured for 96 h with VIP-CALs mixed with anti-VIP Abs (monoclonal c23.5 IgG and isotype-matched nonimmune IgG: IgG from 8 wk old MRL/lpr mice, 20 week old MRL/lpr mice and equivalently aged control MRL/++ mice). Anti-VIP concentrations as small as 10 nM are sufficient to induce decreased secretion of IFN-γ and decreased secretion of IL-4. ELISA methods are used to measure IFN-γ and IL-4 secretion by the T cells. As before, removal of free CAL prior to Ab treatment of the cells allows determination of irreversible VIP-CAL effects. Anti-VIP Ab treatment depletes VIP in T cells, which is thought to disturb autocrine T cell regulation by the peptide. Thus, it is useful to measure the ability of VIP-CALs to restore VIP levels to control levels in the Ab treated cells (by radioimmunoassy of total VIP following dissociation of Ab-VIP complexes at acid pH as in previous studies, ref 47,48). Direct interaction of VIP-CALs with lymphocyte VIP receptors is precluded, as receptor binding requires the entire sequence of VIP (the CALs contain truncated VIP).

These studies allow assessment of comparative bioefficacy of individual DNA-CALs and VIP-CALs. As noted previously, CAL combinations may be necessary to achieve efficient Ab covalent blockade. The ability to covalently saturate the anti-DNA Abs help establish the importance of Ab combining sites in the cellular cytotoxicity and immunohistochemistry studies (as opposed to Fc receptor binding and complement activation mechanisms).

14. Beneficial effects of DNA-CALs. The clinical course of lupus in MRL/lpr mice shares similarities with human lupus. Many hallmarks of human lupus are observed in these mice, e.g., formation of Abs to various autoantigens, kidney damage, proteinuria, multi-organ involvement and heightened T helper cell activity. Abs to DNA and VIP appear spontaneously in the serum of MRL/lpr mice. Thus, the MRL/lpr model is a suitable first approximation to determine the potential ameliorative effects of CALs. DNA-CALs can be administered to MRL/lpr mice at two ages: 8 wks and 14 wks. Serum Abs to certain autoantigens begin to appear at 8 wks and most Abs characteristic of lupus are detected by 14 wks, when kidney damage is overt. Mortality increases rapidly after age 16 wks, and 50% mortality occurs at 22-24 wks. Initially, it is appropriate to study 3 intravenous administrations of DNA-CALs, e.g., at 2 week intervals. Control mice receive diluent. Groups of 8 mice each in the 8 wk cohort are euthanized at 2 wk intervals for detailed analysis until 26 wks (8 mice/group; 10 groups, including the 8 wk baseline group). Similarly, groups of 8 mice each in the 14 wk cohort are euthanized at 2 wk intervals until 26 wks (7 groups including the 14 wk baseline). In addition, eye bleeds are drawn 1 day after each CAL administration for study of acute CAL effects on Ab binding and catalytic activities (see below). CAL doses are adjusted based on Aim 1/2 data (assuming a distribution volume of ~10 ml), which are

predicted to indicate that nM CAL concentrations are sufficient in inhibit the anti-DNA Abs. A mixture of the dsDNA-CAL/oligo-CAL is studied initially to obtain inhibition of diverse Ab subtypes. Subsequent studies include analysis of multiple concentrations of the CALs administered separately as needed. Serum anti-dsDNA/ssDNA titers are measured by ELISA. Reduced anti-DNA titers are predicted (unless anti-DNA Ab replenishment occurs more rapidly than their covalent blockade). Residual DNase and DNA-CAL binding activities of serum IgG is measured as before. The DNA-CALs are not anticipated to serve as immunogens for additional anti-dsDNA Ab synthesis, as elicitation of these Abs requires coimmunization with DNA binding proteins and adjuvant (72). More importantly, covalent DNA-CAL binding by B cell surface Ig is hypothesized to induce immunological anergy – previously, B cell anergy induced by saturating surface Ig with excess antigen has been documented (37,37a). This is tested by estimating the number of DNA-binding B cells in splenocyte preparations by flow cytometry following CAL administration (biotinylated dsDNA and single stranded oligonucleotide probes devoid of phosphonate esters; streptavidin-peroxidase to identify DNA bound to the cells). Staining for anti-CD19 conjugated to phycoerthyrin identifies the B cell subpopulation. Confirmatory studies can be done as needed using ELISASPOT methods, in which B cells are stimulated by the DNA-CAL and DNA in vitro to determine downregulation of Ab synthetic responses attributable to in vivo CAL treatments.

Anti-DNA immune responses are theorized to initiate lupus (e.g., 73). For example, Ab responses to several other autoantigens may be explained by 'epitope spreading' phenomena, in which the presence of cells sensitized to DNA can facilitate Ab responses to proteins associated with DNA. Thus, it is useful to assess the level of the overall immune responses in DNA-CAL treated mice. This is done by measuring total levels of serum immune complexes and rheumatoid factor by standard methods. Study of glomerular damage is by histological examination by an expert pathologist using standard procedures for assessing human biopsies (WHO classification, semiquantitative index), paying attention to the level of cellular hypertrophy, glomerular sclerosis/scarring, excessive extracellular matrix, and presence of inflammatory cells. Proteinuria is read with Chemstrips. Ab and C3 deposition in kidney sections will be by staining with anti-mouse Ig/anti-mouse C3 labeled with FITC, with consideration given to the magnitude and site of staining (e.g., basement membrane, subepithelial spaces, cell surfaces, nuclei). The general health and mortality is recorded by daily inspection of the mice. Once initial data are in hand, repeat experiments can be carried out as needed with sufficient numbers of mice for the study to be statistically powered to enable detection of 20% improvement in mortality.

15. VIP-CAL studies. In vivo VIP-CAL studies are designed essentially as described for the DNA-CALs. Key features are: ***(a)*** Serum anti-VIP Abs binding and catalytic activities are measured by ELISA and radioassay, respectively, following VIP-CAL administration; ***(b)*** Total VIP levels in serum and spleen extracts are determined by radioimmunoassay (47,48); ***(c)*** Flow cytometry studies is done to determine whether VIP-CAL administration induces reduction of B cells that recognize VIP and VIP-CAL – this will help determine whether covalent binding downregulates Ab synthesis; ***(d)*** serum levels of total IgG, anti-DNA Abs, rheumatoid factor and immune complexes are measured for immunochemical correlation; ***(e)*** Flow cytometric determination of T cell subpopulations (CD4+, CD8+, activated CD4+ cells, total B cells (CD19) and NK cells (DX1.1) is done – reduced numbers of activated T helper cells will indicate the overall beneficial effects of VIP-CAL treatment; and ***(f)*** kidney histology, Ab deposition and survival data will be obtained. As in the case of the DNA-CAL studies, a combination of the VIP-CALs is administered initially.

Even if CAL half-life is on the order of hours, beneficial effects can be anticipated. Provided sufficient initial CAL concentrations are obtained, the reaction with Abs should be complete quickly. In addition, if covalent BCR engagement drives B cells into apoptosis, the CALs will inhibit Ab synthesis, precluding any need for prolonged treatment. The CALs are unlikely to serve as immunogens. Elicitation of anti-dsDNA is generally very difficult, although anti-ssDNA binding can be produced when the immunogen is properly formulated. Similarly, VIP administration does not stimulate anti-VIP synthesis, except when the peptide is derivitized with carrier proteins and injected in conjunction with strong adjuvants. As complement activation by anti-DNA Abs is thought to be important in the pathogenesis of lupus, covalent blockade with DNA-CALs may provide only partial benefit. This is not a limitation, if the CALs also inhibit Ab synthesis. Provided study of disease amelioration is accompanied by careful analyses of anti-VIP covalent blockade, an unambiguous conclusion about anti-VIP pathogenicity should be available. Depending on the results of MRL/lpr studies, other lupus-prone mouse strains can be analyzed prior to consideration of humans studies (NZB/W F1; BXSB).

[redacted]

Covalently Reactive CD4 Analogs

Activated nucleophilic amino acids are established to be responsible for covalent catalysis by enzymes such as serine proteases. In recent unpublished studies using biotinylated phosphonate esters, we observed that certain nonezymatic proteins express similar nucleophilic reactivity, including the HIV coat protein gp120. In related studies using nucleophilic antibodies, we were able to efficiently deliver the phosphonate ester moiety to the active site nucleophile by incorporating the phosphonates into peptides and proteins. Such covalently reactive peptide and protein analogs (CALs) serve as specific ligands for the cognate nucleophile in the binding protein on account of non-covalent, high affinity CAL recognition. This approach holds the potential of efficient and permanent blockade of the biological activity of the targeted protein via formation of a covalent bond between the phosphorus and the nucleophile. HIV infection of host cells is initiated by binding of gp120 to CD4 receptors. Noncovalent delivery of CD4-mimetic CALs to the gp120 nucleophile(s) should allow potent and irreversible inhibition of HIV infectivity.

I. Background

1. Nucleophilic protein reactivity: The overall background is depicted in Figs 1 and 2. Protein nucleophilic reactivities derive from chemical activation of the side chains of certain amino acids. In serine proteases, for instance, the precise spatial positioning of the Ser-His-Asp triad allows the formation of a hydrogen bonding network that imparts nucleophilic reactivity to the Ser oxygen This type of nucleophilic reactivity has been assumed until now to be a unique characteristic of enzymes that form covalent reaction intermediates in the course of catalyzing chemical reactions, e.g., certain proteases, glycosidases, lipases and synthases. Once the covalent acyl-enzyme intermediate is formed, it must be hydrolyzed by a water molecule to regenerate the active enzyme and complete the catalytic reaction cycle.

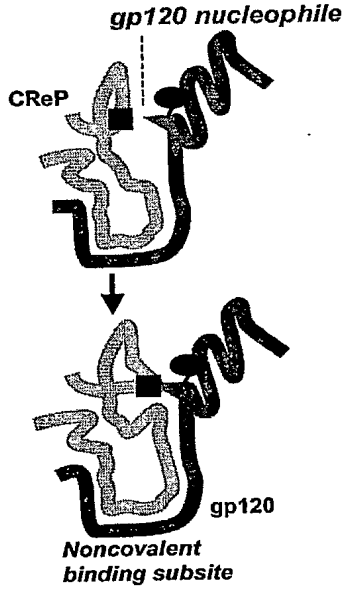

Fig 1. Two step model of CAL ligand recognition by subsites responsible for noncovalent and nucleophilic binding. Initial noncovalent binding does not involve the gp120 nucleophile. Intramolecular interactions impart nucleophilicity to certain residues (triangle). The nucleophile then forms covalent complexes with the ligand at the covalently reactive phosphorus (rectangle) located distant from the noncovalent ligand binding site.

We observed recently that non-enzymatic proteins can also express nucleophilic covalent reactivity. The reactivity was identified using phosphonate diester probes, which are well established inhibitors of serine protease enzymes and catalytic antibodies (1,2). The phosphorous atom in these compounds expresses electrophilic character, and depending on the strength of the leaving group at the ester bonds, the phosphonates can form stable covalent complexes with the chemically activated nucleophilic residues. In preliminary studies, the following proteins were observed to form covalent complexes with biotinylated phosphonate diesters that remained stable to denaturing conditions: several noncatalytic antibodies, gp120, albumin and ovalbumin. gp120 displayed the greatest nucleophilic activity among the non-antibody proteins. On a molar basis, the level of nucleophilic activity of gp120 is at least 10-fold greater than of trypsin. As the latter protein is highly evolved to maximize its covalent reactivity, the observed reactivity of gp120 is unlikely to be a trivial phenomenon. Although gp120 has no known enzymatic activity, its membership in the immunoglobulin superfamily is relevant (3), as the immunoglobulins express exceptional levels of nucleophilic reactivity.

Mechanistic considerations of covalent acyl transferase reactions help differentiate between nucleophilic and catalytic proteins, even though both may employ similar covalent mechanisms. The covalent reactivity observed in gp120 is a necessary but not sufficient condition for covalent catalysis. For example, catalytic cleavage of peptide bonds by serine proteases also requires facilitation of events occurring after formation of the covalent acyl-enzyme intermediate, that is, hydrolysis of the intermediate and release of product peptide fragments from the active site (Fig 2). Therefore, only a small subset of nucleophilic proteins is predicted to express catalytic activity. Thus, the observation of covalent reactivity of noncatalytic proteins, although initially

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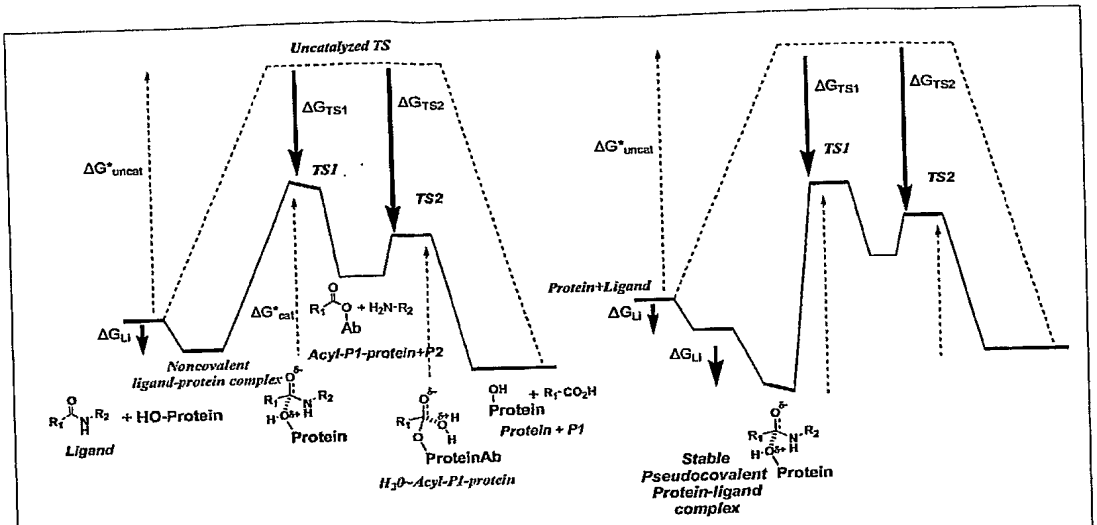

Fig 2: Reaction coordinates for nucleophilic proteins. ***Left,*** Serine proteases stabilize the antigen ground state noncovalently (ΔG_{Ag}). Nucleophiles such as an activated Ser residue attacks the peptide bond, forming an unstable resonant transition state (TS1). Completion of this reaction forms the covalent acyl-Ab intermediate, with release of the C terminal peptide fragment. In the second reaction, a water molecule hydrolyzes the covalent intermediate via a second tetrahedral transition state (TS2). ***Right,*** The reaction proceeds as above, except that the resonant antigen-Ab complex containing partial covalent bonds (TS1 above) is more stable than the ground state of the antigen-Ab complex. $\Delta G^{\dagger}_{uncat}$ and $\Delta G^{\dagger}_{cat}$ correspond to activation energies for the uncatalyzed and catalyzed reactions, respectively. K_m is a function of the extent of ground state stabilization (ΔG_S). k_{cat}/K_m is a function of extent of transition state stabilization relative to the catalyst-substrate ground state complex.

unexpected, does not present any inherent contradiction with established enzymology concepts. As noted in the preceding paragraph, the nucleophilic behavior of gp120 is analogous to that of several antibodies without known enzymatic activity.

Previously, covalent protein-phosphonate interactions have been held analogous to resonant covalent structures found in the transition state and acyl-enzyme intermediate formed en route to catalytic peptide and ester bond hydrolyses reactions (Fig 2). We believe that noncatalytic nucleophilic proteins form weak covalent bonds in which resonant structures can stabilize the antigen ground state (Fig 2, right) . [A covalent bond is one in which electrons are shared in a common orbital; although covalent bonds are usually stronger than noncovalent bonds, the strength of these bond is not a defining feature of a covalent bond – a hydrogen bond, for example, has partial covalent character]. According to this proposal, nucleophilic proteins hold the potential of forming more stable, dead-end pseudocovalent complexes with the ligand. Examples of noncatalytic antibodies forming covalent complexes with antigens have been published (4). This type of bonding may also be involved in formation of certain stable protein-protein aggregates, such as amyloid fibrils of antibody light chains and β-amyloid peptide. These aggregates are often stable to denaturing conditions, but no systematic basis for understanding their stability based on noncovalent binding forces is available.

II.2. Message-address system for covalent gp120 inhibition.

High affinity gp120 binding to host cell CD4 receptors initiates HIV infection. The approach of covalent inhibition of gp120-CD4 binding has the advantage of permanent virus inactivation. Conventional strategies such as noncovalent inhibition by soluble CD4 or noncatalytic antibodies to gp120 (and CD4), on the other hand, allow regeneration of biologically active gp120 and CD4 upon dissociation of the noncovalent complex. Simple phosphonate diesters described in the preceding paragraph are not useful to achieve covalent inhibition of the virus, as they also react with enzymes and other proteins. This problem can be overcome by incorporating the phosphonate moieties within peptides and proteins. Essentially, such covalently reactive peptides and proteins (CALs) serve as a message-address system in which the peptide determinant serves as the address whereby the phosphonate group (message) can be delivered to the active nucleophilic amino acids of the target protein. The feasibility of this approach has been established using the following CALs to obtain covalent binding to antibodies: a gp120 peptide containing the phosphonate ester at the C terminus, full-length gp120 derivitized at Lys side chains with the phosphonate ester, and the epidermal growth factor receptor derivitized with the phosphonate ester (5, Preliminary Studies). The strategy may be generally applicable to any protein targeted by a CAL

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ligand, provided the following conditions are met: *(a)* noncovalent interactions of the protein with a determinant in the CAL allows high affinity binding; *(b)* the targeted protein contains a nucleophile that is sufficiently reactive with the phosphonate, and *(c)* the protein nucleophile and the phosphorous atom are in register with each other spatially.

The utility of noncovalent binding as a delivery mechanism for achieving covalent inhibition is evident from preliminary studies on nucleophilic antibodies. CAL binding by antigen-specific antibodies was observed to occur at levels ~ 4 log orders greater than simple phosphonate ester binding by trypsin (see Preliminary Studies). Nonspecific CAL binding by nonimmune antibodies and irrelevant proteins is negligible. We believe that one or more nucleophilic binding interactions are critical to stabilization to the gp120-CD4 complex. Therefore, the CD4 binding site of gp120 should contain a nucleophile to which the phosphonate can bind covalently. According to the split site model of nucleophilic gp120-CD4 binding, sufficient flexibility in the active site of gp120 and the CAL peptide determinant exists to allow appropriate spatial positioning of the nucleophile and the phosphorus atom (Fig 1). This model has proved useful in understanding how nucleophilic proteins can combine noncovalent ligand recognition with the covalent reaction (Fig 1; 6). Active site nucleophiles are observed to be functionally coordinated with noncovalent protein-ligand interactions in all antibodies examined thus far. Mutations at the catalytic residues of an antibody did not interfere with initial antigen ground state recognition and the site of peptide bond cleavage is distant from the noncovalently recognized epitope (6). This has lead to the hypothesis that distinct subsites are responsible for the initial noncovalent binding and the subsequent covalent reaction steps. In this model, the nucleophile makes little or no contact with the ligand until after the noncovalent step is complete. Once initial binding has occurred, flexibility in the active site allows the nucleophile to complete the chemical reaction.

A model of the CD4-gp120 complex has recently been constructed based on the X-ray structure of a truncated version of gp120 complexed with CD4 and the Fab fragment of an antibody (7,8). The model suggests that the gp120 binding site of a CD4 is a discontinuous determinant composed of amino acids that are distant from each other in the linear sequence. Additional studies have highlighted the importance of backbone flexibility in the binding, including formation of new peptide determinant(s) important for chemokine receptor binding as a consequence of the initial gp120 interactions with CD4. Certain linear peptides derived from CD4 bind the monomer gp120 as well as the native protein on the viral surface, but their binding affinities are not impressive. Among small peptide CD4 analogs, a mimetic containing certain CD4 contact residues embedded in a scyllatoxin scaffold appears to bind gp120 with highest affinity (8). Soluble CD4 purified from CHO cells is thought to bind gp120 with affinity comparable to cellular CD4 (8,9).

Covalent gp120 binding by the appropriate CAL holds the potential of inducing a global change in the conformation of the protein. In this event, loss of biological activities that reside outside the CD4 binding site of gp120 may be foreseen. For instance CAL binding to gp120 may disrupt its ability to bind gp41, inducing its release from the viral surface. Such fortuitous effects will be beneficial from the viewpoint of reducing viral infectivity.

II. Examples relevant to CD4-CALs

1. CAL validation using nucleophilic antibodies: A message-address system was devised to isolate catalytic Abs to gp120 (Fig 3; ref 5). Essentially, binding at the noncovalent site (address) is employed as the means to deliver the peptide to the antibody nucleophile (message). Lupus patients express antibodies to a conserved gp120 peptide determinant (residues 421-436). We synthesized gp120(421-431) with a phosphonate diester group in place of K432 at the C terminus and biotin at the N terminus (tCAL). To isolate catalysts, phages from lupus Fv and L chain libraries (1) were selected by covalent tCAL binding. Two L chains with biotinylated gp120 cleaving activity were identified (biotin attached to Lys side chains of purified gp120, strain SF2, using a bifunctional cross-linker). One L chain has been characterized further (clone SKL6, GenBank number AF522074). This L chain contains 3 replacement mutations compared to its germline V gene counterpart O2/O12 (located in framework 1, CDR 1 and CDR2; Arg18:Gly,Asn34:His, Ala50:Val). gp120 cleavage by the L chain occurs with a submicromolar Km value. No cleavage of biotinylated BSA or the biotinylated extracellular domain of the epidermal growth factor receptor (exEGFR) was evident by electrophoresis procedures similar to those employed for Bt-gp120 (Bt-BSA from Sigma; exEGFR from M. O'Connor; biotinylated at Lys side chains, 3 mol biotin/mol protein). Covalent tCAL binding by the L chain was inhibited by gp120(421-436) but not by equivalent concentrations of an irrelevant peptide (synthetic EGFR peptide 294-310), indicating active site protection due to noncovalently bound antigen. Time-dependent accumulation of tCAL-L chain adducts was evident. The second order rate constant kapp/[tCAL] value deduced from the saturation curve was 1.60 M^{-1} sec^{-1} compared to a value of 0.06 M^{-1} sec^{-1} for trypsin. Thus, the overall efficiency of the nucleophilic reaction with the L chain is superior to that with trypsin. Adduct formation was inhibited by

disopropylfluorophosphate (DFP) and no adduct formation was observed using proteins denatured by heat/SDS treatment prior to incubation with the tCAL. Similar conclusions were obtained using noncatalytic antibodies to gp120(421-436) and exEGFR (not shown). Polyclonal IgG raised against gp120(421-436) was analyzed for covalent binding to the peptidyl gp120(421-431)tCAL. Electrophoresis of IgG treated with the tCAL indicated saturable accumulation of the 153 kD covalent adducts (IgG, 150 kD; tCRA, 2.2 kD). The second order rate constant (kapp/[tCAL]) computed from the saturation was plot is 18.4 M^{-1} sec^{-1}, compared to a value of 0.06 M^{-1} sec^{-1} for trypsin binding by the tCAL. Covalent tCAL binding by nonimmune IgG was virtually undetectable. Formation of the adducts was inhibited by treatment of IgG with gp120(421-436)-BSA but not BSA, indicating that the covalent reaction must occur close to the noncovalent peptide binding site. Studies on proteins in which phosphonate diester groups are placed on Lys side chains have yielded interesting data despite unpredictable spatial positioning of the phosphonate with respect the peptide backbone [as well as unpredictable phosphonate availability in the epitopes recognized by the antibodies]. SDS-electrophoresis of biotinylated exEGFR-pCAL (3 mol biotin and 15 mol phosphonate/mol exEGFR) yielded a single silver stained band at 85 kD. Polyclonal IgG raised to EGFR formed covalent complexes with Bt-exEGFR-pCAL complexes, evident from saturable accumulation of the 235 kD band (IgG, 150 kD; exEGFR-pCRA, 85 kD). The 235 kD adduct band was stainable by an anti-mouse IgG-peroxidase conjugate in immunoblots. Covalent Bt-exEGFR-pCAL binding by nonimmune IgG was not detected. Formation of the adducts was inhibited by treatment of IgG with exEGFR, indicating that covalent binding occurs within the Ab combining sites. The overall covalent binding efficiency assessed from the second order rate constant was 904 M^{-1} sec^{-1}, compared to a value of 0.1 M^{-1} sec^{-1} for trypsin binding. Three commercially available anti-exEGFR monoclonal antibodies were analyzed for Bt-exEGFR-pCAL covalent binding (Labvision, CA). All 3 formed 235 kD adducts at varying levels. No adducts were formed by a control monoclonal antibodies to BSA. These studies indicate functionally coordinated nucleophilic and nonocovalent binding activities of the antibodies.

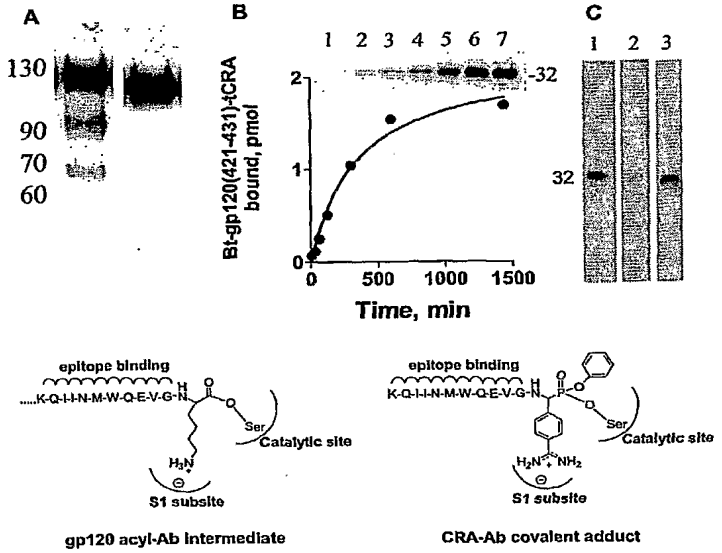

Fig 3. Characteristics of lupus L chain clone SKL6 isolated by phage binding to gp120(421-436)tCAL. *A,* SDS- electrophoresis gels showing gp120 cleavage by light chain clone SKL6 (left lane) and control gp120 treated with a noncatalytic L chain (right lane). gp120 (SF2) biotinylated at Lys residues (0.3 mol biotin/mol gp120; 0.03 μM); SKL6 0.03 μM, 21 hours incubation. Blot stained with streptavidin-peroxidase. ***B,* Covalent L chain binding of Bt-gp120(421-431)tCAL as a function of time.** L chain 1 μM, tCAL 10 μM. Inset, streptavidin-peroxidase stained blots of SDS-gels corresponding to the time points in the graph. ***C,* Active site protection by gp120(421-436).** Shown are SDS gels of covalent Bt-gp120(421-431)tCAL (10 μM) adducts formed with the L chain (1 μM) in the absence (lane 1) and presence of gp120(421-436) (10 μM; lane 2) and an irrelevant peptide [EGFR(351-364), 10 μM; lane 3]. ***Bottom,* Schematic diagrams showing design elements underlying CAL mimicry of Ab interactions with the peptide antigen.**

2. gp120 nucleophilicity: Two lines of evidence indicate the nucleophilic reactivity of gp120: ***(a)*** its reaction with simple phosphonate diesters, and ***(b)*** formation of covalent oligomers by gp120 derivitized at Lys side chains with the phosphonate diester. Incubation of the recombinant gp120 with the biotinylated phosphonate diester resulted in formation of covalent gp120-phosphonate adducts stable to denaturing conditions (boiling, TCA precipitation, 2% SDS) (Fig 4). Under equivalent conditions, formation of trypsin-phosphonate occurred at 15.7 fold lower rate. Albumin and ovalbumin also formed phosphonate adducts visible as 68 kD and 44 kD bands, but at even lower rates. No adduct formation with the extracellular domain of α-lactalbumin, calmodulin, soybean trypsin inhibitor or ribonuclease was evident at concentrations up to 5 μM protein and 100 μM biotinylated phosphonate ester.

Gp120 derivitization at Lys side chains with the phosphonate diester and introduction of biotin at additional Lys side chains was done as in the preceding section. The resulant preparation, designated gp120-pCAL, displayed a propensity to form covalent oligomers upon incubation at 37°C in a neutral pH buffer (Fig 4). Under similar conditions, no oligomerization of control EGFR-pCAL or albumin-pCAL was observed. Electrophoresis of the reaction mixture under reducing

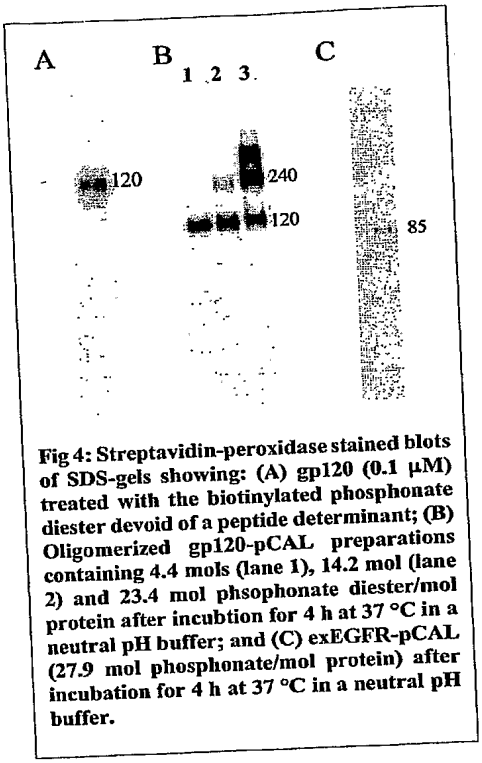

Fig 4: Streptavidin-peroxidase stained blots of SDS-gels showing: (A) gp120 (0.1 μM) treated with the biotinylated phosphonate diester devoid of a peptide determinant; (B) Oligomerized gp120-pCAL preparations containing 4.4 mols (lane 1), 14.2 mol (lane 2) and 23.4 mol phsophonate diester/mol protein after incubtion for 4 h at 37 °C in a neutral pH buffer; and (C) exEGFR-pCAL (27.9 mol phosphonate/mol protein) after incubation for 4 h at 37 °C in a neutral pH buffer.

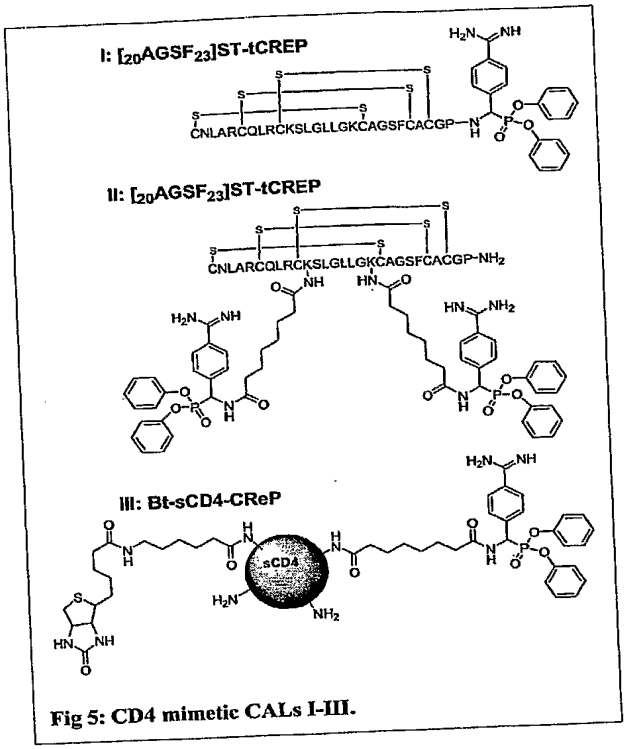

Fig 5: CD4 mimetic CALs I-III.

conditions did not affect the mass of the oligomers, indicating that the S-S bonding is not a factor in oligomerization. Increasing oligomerization was observed with increasing derivitization of gp120 moles by the phosphonate diester moeities (Fig 4B). Taken together, these observations suggest that the oligomerization reaction occurs by covalent binding of gp120 nucleophile(s) with the phosphonate ester.

3. Reagents and methods available for CAL validation. Under NIH program project AI46029, we have developed various reagents/methods needed for the present project. These include: ***(a)*** neutralization assays using primary isolates from all known subtypes of HIV and PBMC hosts (collaboration with Dr. Carl Hanson, California Public Hlth Lab, Berkeley); ***(b)*** organic/peptide chemistry methods to synthesize peptidic/proteinic phosphonates and characterize them by mass spectrometry, NMR, ELISA and electrophoresis; and ***(c)*** various antibodies to gp120 and CD4.

4. CAL preparation: The overall strategy is: ***(a)*** Target the conserved CD4 binding site of gp120 using CD4-derived CALs; ***(b)*** Analyze covalent binding of the CALs by monomer gp120 and intact HIV; and ***(c)*** Determine the potency with the CALs and their nocovalent counterparts inhibit HIV infection. Examples of CALs are given in Fig 5: ***(a)*** soluble CD4 (sCD4) derivitized at Lys side chains with phosphonate ester groups; ***(b)*** a CD4 peptide mimetic with a phosphonate ester located at the C terminus, and ***(c)*** the CD4 peptide mimetic with phosphonate ester groups located at Lys side chains. sCD4 protein is composed of residues 1- 369 of CD4 (8,9). Similarly, the CD4 mimetic peptide has been selected for CAL preparation because it binds gp120 with comparatively high affinity (8) compared to other peptide analogs and mimotopes of the CD4 binding site of gp120. Trials of a soluble form of CD4 for therapy have been conducted, but success has been limited, presumably because the the sCD4 does not saturate viral gp120. CALs are designed to bypass a major limitation of the gp120 binding site mimetics, that is, recovery of viral infectivity upon dissociation of the noncovalent complex. In the reaction scheme of Fig 6, the goal is to maximize covalent inactivation of gp120 by exploiting the noncovalent and covalent binding features of the CALs (low Ki, high k3). Basic structural elements of the CALs are: ***(a)*** the peptidic determinants of CD4 allowing noncovalent binding to gp120; ***(b)*** strong leaving groups in the phosphonate diester (phenols), designed to increase the covalent reactivity of the phosphorus; and ***(c)*** the positively charged amidino group, which serves as a Lys/Arg mimetic, corresponding to the P1 specificity observed for gp120 previously (not shown). Biotin is incorporated at the N terminus of the peptides or at Lys side chains to monitor the binding reaction.

Simple phosphonate ester syntheses have been described previously (1,2). Standard chemical synthesis techniques will be applied to prepare the CALs **I-III** (e.g.,5). The peptidic component in CALs **I** and **II** is identical, i.e., CD4 contact residues located in a scyllatoxin scaffold. CAL **I** is be prepared by solution phase synthesis of the phosphonate diester unit combined with solid phase peptide synthesis and purified by preparative HPLC. The phosphonate diester moiety is attached via a dicarboxylic acid linker to the N terminus of the peptide. CAL identity is confirmed by mass spectroscopy and NMR. CAL II is obtained by conjugating diphenyl amino(4-amidinophenyl)methanephosphonate and the biotinylated peptide using the commercially available homo-bifunctional linker, bis-sulfosuccinimidyl suberate, followed by HPLC separation. The position of the acylated Lys is determined by MS/MS analysis of the product (this procedure generates daughter ions by ordered fragmentation of the parent molecular ion, allowing assignment of the phosphonate to individual amino acids). The whole protein CAL **III** is prepared essentially as CAL **II** using recombinant soluble CD4 (residues 1-369) purified from CHO cells (8). The protein derivitized at Lys residues with the phosphonate and biotin moieties is subjected to SDS-electrophoresis (expected mass ~ 55 kD), and the extent of derivitization is determined by measuring the amount of biotin in blots using biotin-albumin as standard. A BioRad imaging system is used for quantification, taking care to remain in the linear range of the standard curve.

$$\text{gp120} + \text{CLA} \overset{K_i}{\rightleftharpoons} \text{gp120}\sim\text{CLA} \xrightarrow{k_3} \text{gp120-CLA}$$

Fig 6. Reaction scheme for initial noncovalent binding of a CAL by gp120 and the subsequent covalent reaction. Hydrolysis of the covalent gp120-CAL complex is assumed to be negligible.

5. Covalent gp120 binding: The suitability of the CALs as gp120 inhibitors is determined by measuring the Ki and k3 for their covalent binding by monomer gp120 and the protein on the surface infected cells (which serves as a model for the native gp120 on the viral surface). Increasing concentrations of the CALs are incubated with suitable amounts of the purified protein (SF2) or infected cells (H9 cells, SF2 strain). The reaction mixtures are analyzed by SDS-electrophoresis with detection of the biotinylated CALs using streptavidin peroxidase as in previous studies gp120 adducts of CAL **I** and **II**, ~122 kD; gp120-CAL **III** adduct, 175 kD). In the case of the infected cells, the gp120 is extracted using the detergent CHAPs and the gels are also stained with anti-gp120 antibodies to confirm that gp120 is present in the biotin-containing adducts. If needed, the adducts can be purified by immunoaffinity chromatography on immobilized anti-gp120 prior to electrophoresis. The equilibrium dissociation constant Ki and the first order rate constant for covalent inhibitor binding k3 are measured according to Kitz-Wilson (2), which provide a full kinetic description of the reaction [accumulation of covalent adducts is determined as a function of time at several CAL concentrations]. Ki is a measure of the strength of noncovalent binding, and k3, the strength of the nucleophilic reactivity. Substrate specificity is analyzed using a panel of commercially available proteins (calmodulin, EGFR, albumin) – no adduct formation should occur if the CALs are specific for gp120.

6. HIV neutralization: Increasing concentrations of the three CALs and controls (underivitized peptide $[_{20}AGSF_{23}]ST$ and underivitized sCD4, Fig 5) are analyzed for neutralization of HIV infection using peripheral blood mononuclear cells as hosts as in our previous studies conducted according to ref 10. Release of p24 is monitored as index of the level of infection using an enzyme immuoassay. Initial studies are done using a clade B primary viral isolate (strain 23135) that affords sensitive detection of neutralizing activity by antibodies directed to the CD4 binding site of gp120. As a conserved site in gp120 is targeted by our approach, broad neutralizing activity is anticipated, which is analyzed using additional strains derived from clades A, C, D and E. The CALs should neutralize HIV with potency greater than the control peptide and soluble CD4. Irreversible inhibition by the CALs is predicted. Thus, washing of CAL-treated HIV should not allevate the inhibition, whereas HIV treated with the control peptide/sCD4 is predicted to recover its infectivity.

7. Prospects for CALs therapy: To help design superior CALs, additional studies can be undertaken to identify the gp120 nucleophile(s). This can be done by mass spectroscopy of CAL-labeled gp120 tryptic fragments, as in our previous studies on trypsin and antibodies. The covalent reactivity of the CALs can be improved by using superior leaving groups, e.g., p-nitrophenol, and flanking residue specificity can be improved by empirical studies in which the amidino group and various amino acids are replaced by other analogs/amino acids. Additional factors important for therapy are the stability of the CALs in vivo and their ability to penetrate lymphoid organs, which can harbor large amounts of HIV.

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Submitted

Covalent gp120 oligomers for vaccination and antibody generation.

Herein are the background and examples pertaining to formation covalent gp120 trimers guided by noncovalent self-assembly of the protein and to elicit Abs to covalently stabilized trimeric gp120 that broadly inhibit primary isolates of HIV.

Background

1. Mechanisms and limitations of neutralizing antibodies: Many experimental HIV vaccines elicit antibodies which bind to, but fail to neutralize, diverse strains of HIV. Attempts to use HIV antibodies for passive immunotherapy have similarly failed to inhibit the broad spectrum of HIV clinical strains. It has been theorized that high affinity may define the small subset of functional neutralizing antibodies [1-4]. Neutralization of viruses by antibodies is determined by in vitro tests, and is defined simply as reduction of infectious titer in the presence of the antibody. Thus, possible traditional mechanisms include (1) antibody blocking of the virus's host-cell binding site; (2) an antibody-induced allosteric change in overall virus conformation; (3) prevention of virus uncoating by antibody after entry of the antibody-coated virus into the host cell; (4) antibody-induced aggregation of infectious viruses; and (5) effects of antibody directed against host-cell components involved in the infectious process. By binding to non-neutralizing epitopes on the viral surface, antibodies may also increase virus infectivity titers. Possible mechanisms of this antibody-dependent enhancement (ADE) include (i) enhanced interaction with complement receptors on host cells, when complement has bound to the antibody-virus complex, and (ii) interaction of virus-antibody complexes with antibody F_C receptors on host cells. Finally, neutralizing and/or enhancing antibodies in polyclonal antibody mixtures may have synergistic or antagonistic interactions which govern the net observed degree of neutralization or enhancement. Non-neutralizing antibodies may inhibit HIV infection by mediating antibody dependent cellular cytotoxicity (ADCC), and, while not "neutralizing" in the classical sense, antibodies may block spread of viral infection by blocking cell-to-cell fusion. Antibodies directed against normal host cell surface molecules may also block viral infection before or after absorption of the virus. Non-immunoglobulin factors in plasma may also inhibit HIV infection, thus confounding interpretation of in vitro neutralizing antibody assays but possibly contributing to control of infection in vivo.

Experimental HIV vaccine design has been heavily influenced by the observation that in acute infection neutralizing antibodies do not appear until viral load appears to be waning under the influence of cytotoxic T cell responses. The recent observation that neutralization of primary isolates detected on primary macrophages, rather than on traditional lymphocytes, comes up much earlier after seroconversion [6] , on the other hand, gives impetus to the design of HIV vaccines capable of eliciting neutralizing antibodies.

Earlier work with T-cell line adapted (TCLA) HIV gave the misleading impression that HIV is highly sensitive to neutralization. Recently interest has shifted to neutralization of non-syncytium-inducing, monocytotropic, primary clinical isolates of HIV, believed to be most relevant to transmission and to targeting in post-exposure immunoprophylaxis. These latter viruses are relatively refractory to neutralization by immune sera, monoclonal antibodies, and soluble CD4 [7-11]. Both genetic and epigenetic mechanisms for this resistance remain under active investigation [12, 13]. As studied in such assays, immunization of normal human volunteers with experimental HIV vaccines has induced disappointingly low levels of neutralizing antibodies (to other than the immunizing HIV strain).

2. Passive immunotherapy and immunoprophylaxis for HIV disease: Passive administration of HIV specific antibodies has correlated with modest therapeutic benefit in several small clinical trials [14]. Limitations on further development of this approach and its extension to immunoprophylaxis, however, include: (1) in the case of polyclonal antiserum: limited sources, difficulty of reproducible production, and safety concerns; (2) in the case of monoclonal antibodies (MAbs): a failure to demonstrate neutralization of a broad range of primary clinical isolates, and (3) in both cases: the irreproducibility of assays for determining such neutralization, a lack of understanding of the in vitro correlates of clinical efficacy, and concern over potential adverse immunopotentiation of HIV infection. A related, but relatively overlooked, approach to immunotherapy and post-exposure immunoprophylaxis is the administration of antibodies targeting HIV receptors and coreceptors. Here, the major limitation is concern over potential immunosuppression by such treatment.

Anti-retroviral agents from three classes of drugs are available for the treatment of HIV infection. Such highly active antiretroviral treatment (HAART) regimens are complex, have major side effects, pose difficulty with adherence, and carry serious potential consequences for the development of viral resistance. Furthermore, as pointed out by David Ho, the use of current drug regimens is extremely unlikely to result in the eradication of HIV in an individual because of the slow decay of "latent" HIV reservoirs in resting T memory cells. The only hope is combination anti-viral

therapy so potent that HIV replication, and hence mutation, is suppressed in even the slowest replicating compartments. Toxicity and difficulty adhering to complicated dosing regimens limit this approach. However, the continuous presence of antibodies that block the infection of new cells while infected cells die off and are replaced could break the chain of viral replication. Passive immunization would be an "orthogonal" treatment to HAART drugs and should synergize with them when used in combination. Toxicity of an antibody, if any, will not enhance toxicity of anti-retroviral drugs and patient adherence to a regimen of an occasional treatment with antibody will be high. Antibody infusions, including the proposed use of anti-viral monoclonal antibodies and clinical trials with HIV immune sera (HIVIG) for the treatment of HIV infection has been an active area of research [14]. However, progress towards development of antibodies for HIV has been impeded by the low neutralization potency of most anti-HIV antibodies and anti-sera against clinical isolates [15] and by neutralization-resistant isolates [16]. Viral variation, recently described by putative neutralization serotypes related to phenotype rather than genetic subtype, also remains as an obstacle to the development of anti-viral antibodies [16].

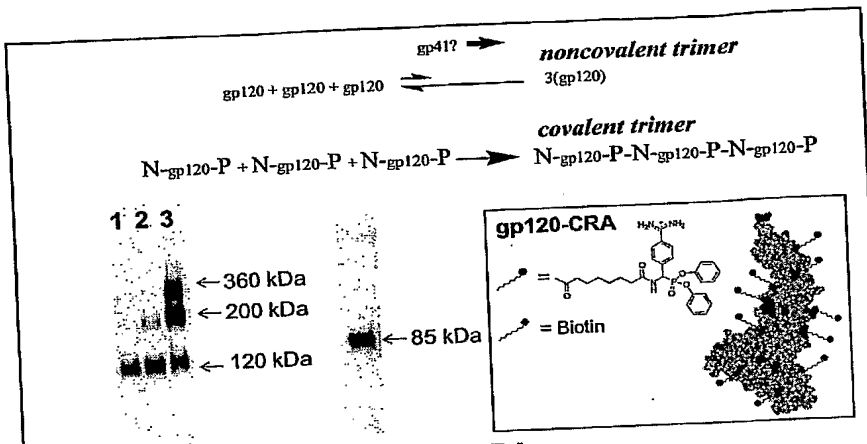
3. Nucleophilic binding interactions: Activated nucleophilic amino acids are established to be responsible for covalent catalysis by enzymes such as serine proteases. In recent studies, we observed that certain nonenzymatic proteins express similar nucleophilic reactivity, including the HIV coat protein gp120. In related studies using nucleophilic antibodies, they were able to efficiently deliver the phosphonate ester moiety to the active site nucleophile by incorporating the phosphonates into peptides and proteins. Such covalently reactive peptides and proteins (peptide CRAs and protein CRAs) serve as specific ligands for the cognate nucleophile in the binding protein on account of non-covalent, high affinity CRA recognition. This can be applied for efficient and permanent blockade of the biological activity of the targeted protein via formation of a covalent bond between the electrophile and the nucleophile.

The overall background is depicted in **Figs. 4 and 5.** Protein nucleophilic reactivities derive from chemical activation of the side chains of certain amino acids. In serine proteases, for instance, the precise spatial positioning of the Ser-His-Asp triad allows the formation of a hydrogen bonding network that imparts nucleophilic reactivity to the Ser oxygen This type of nucleophilic reactivity has been assumed until now to be a unique characteristic of enzymes that form covalent reaction intermediates in the course of catalyzing chemical reactions, e.g., certain proteases, glycosidases, lipases and synthases. Once the covalent acyl-enzyme intermediate is formed, it must be hydrolyzed by a water molecule to regenerate the active enzyme and complete the catalytic reaction cycle.

We observed recently that non-enzymatic proteins can also express nucleophilic covalent reactivity. The reactivity was identified using phosphonate diester probes, which are well established inhibitors of serine protease enzymes and catalytic antibodies [17, 18]. The phosphorous atom in these compounds expresses electrophilic character, and depending on the strength of the leaving group at the ester bonds, the phosphonates can form stable covalent complexes with the chemically activated nucleophilic residues. In preliminary studies, the following proteins were observed to form covalent complexes with biotinylated phosphonate diesters that remained stable to denaturing conditions: several noncatalytic antibodies, gp120, albumin and ovalbumin. gp120 displayed the greatest nucleophilic activity among the non-antibody proteins. On a molar basis, the level of nucleophilic activity of gp120 is at least 10-fold greater than of trypsin. As the latter protein is highly evolved to maximize its covalent reactivity, the observed reactivity of gp120 is unlikely to be a trivial phenomenon. Although gp120 has no known enzymatic activity, its membership in the immunoglobulin superfamily [19] is relevant, as the immunoglobulins express exceptional levels of nucleophilic reactivity.

Mechanistic considerations of covalent acyl transferase reactions help differentiate between nucleophilic and catalytic proteins, even though both may employ similar covalent mechanisms. The covalent reactivity observed in gp120 is a necessary but not sufficient condition for covalent catalysis. For example, catalytic cleavage of peptide bonds by serine proteases also requires facilitation of events occurring after formation of the covalent acyl-enzyme intermediate, that is, hydrolysis of the intermediate and release of product peptide fragments from the active site Therefore, only a small subset of nucleophilic proteins is predicted to express catalytic activity. Thus, the observation of covalent reactivity of noncatalytic proteins, although initially unexpected, does not present any inherent contradiction with established enzymology concepts. As noted in the preceding paragraph, the nucleophilic behavior of gp120 is analogous to that of several antibodies without known enzymatic activity.

Previously, covalent protein-phosphonate interactions have been held analogous to resonant covalent structures found in the transition state and acyl-enzyme intermediate formed en route to catalytic peptide and ester bond hydrolyses reactions. We believe that noncatalytic nucleophilic proteins form weak covalent bonds in which resonant structures can stabilize the antigen ground state. [A covalent bond is one in which electrons are shared in a common orbital; although covalent bonds are usually stronger than noncovalent bonds, the strength of these bond is not a defining feature of a covalent bond – a hydrogen bond, for example, has partial covalent character].



Bt-gp120-CRA VIb Bt-exEGFR-CRA
CRA/gp120 (mol/mol): CRA/exEGFR (mol/mol): 28
1, 4.4; 2, 14; 3, 23

Fig 1: Covalent trimerization of gp120 derivitized at Lys side chain amines with phosphonate diesters. Streptavidin-peroxidase stained blots of SDS-gels showing oligomerized gp120-CRA preparations containing 4.4 mol (lane 1), 14.2 mol (lane 2) and 23.4 mol phosphonate diester/mol protein after incubation for 4 h at 37 °C in a neutral pH buffer; and lack of an identically prepared EGFR-CRA (27.9 mol phosphonate/mol protein) after incubation for 4 h at 37 °C in a neutral pH buffer. ***Top.*** Association of gp120 into trimers is hypothesized to involve weak forces and occurs very slowly ordinarily (noncovalent gp120 binding to gp41 may accelerate this process). N, nucleophilic residue; P, phosphonate moiety. Formation of covalent trimers of gp120-CRA is hypothesized to occur in a two step process – noncovalent association of gp120 monomers, which brings gp120 nucleophiles into apposition with the phosphonate group, followed by covalent bond formation. As dissociation of the trimers is precluded, their accumulate becomes increasingly evident as a function of time. Correct interfacial apposition of gp120 protomers in the covalent trimers is hypothetical at present, but to the extent that gp120 tends to self-assembles spontaneously into correctly interfaced trimers (aided by gp41), noncovalent binding forces can be visualized to promote proper assembly of the covalent trimers.

According to this proposal, nucleophilic proteins hold the potential of forming more stable, dead-end pseudocovalent complexes with the ligand. Examples of noncatalytic antibodies forming covalent complexes with antigens have been published [20]. This type of bonding may also be involved in formation of certain stable protein-protein aggregates, such as amyloid fibrils of antibody light chains and beta-amyloid peptide. These aggregates are often stable to denaturing conditions, but no systematic basis for understanding their stability based on noncovalent binding forces is available.

4. gp120 multimers: The HIV-1 envelope glycoprotein complex consists of three gp120 and three gp41 subunits anchored in the viral membrane by the gp41 transmembrane region. When used as the basis for experimental HIV vaccines, HIV-1 gp120 monomers have repeatedly failed to elicit effective cross-protective immune responses nor to elicit antibodies capable of effectively binding assembled envelope trimers. Many laboratories have therefore sought to form native trimeric HIV envelope glycoproteins as candidate immunogens. Approaches have included deletion of the proteolytic cleavage site between gp41 and gp120 and deletion of the gp41 intracytoplasmic tail (to give "gp140") in order to allow solubility and avoid nonspecific aggregation. The resulting oligomers were however disappointingly heterogeneous. Some success has been achieved in driving the formation of soluble gp140 trimers by fusing onto the C terminal molecules with trimeric motifs, including GCN4 [21] and T4 bacteriophage fibritin [22]. While these assemblies have been better recognized by potent neutralizaing antibodies such as IgG1b12 and 2G12, the conformations nevertheless appear to be distinct from that of the native fusion-active bundle on the surface of virions [22]. A trimeric structure stabilized by covalent interactions, as proposed herein, would have significant advantages for preparation of a practical immunogen, provided that native conformation is maintained in the covalently stabilized trimer.

Examples relevant to covalent gp120 oligomers

1. gp120 nucleophilicity: Two lines of evidence indicate the nucleophilic reactivity of gp120: ***(a)*** its reaction with simple phosphonate diesters, and ***(b)*** formation of covalent oligomers by gp120 derivitized at Lys side chains with the phosphonate diester. Incubation of the recombinant gp120 with the biotinylated phosphonate diester resulted in formation of covalent gp120-phosphonate adducts stable to denaturing conditions (boiling, TCA precipitation, 2% SDS) (**Fig. 1**). Under equivalent conditions, formation of trypsin-phosphonate occurred at 15.7 fold lower rate. Albumin and ovalbumin also formed phosphonate adducts visible as 68 kD and 44 kD bands, but at even lower rates. No adduct formation with the extracellular domain of lactalbumin, calmodulin, soybean trypsin inhibitor or ribonuclease was evident at concentrations up to 5 uM protein and 100 uM biotinylated phosphonate ester.

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$$\text{Gp120} + \text{CRA} \overset{Ki}{\leftrightarrow} \text{gp120}\sim\text{CRA} \overset{k_3}{\rightarrow} \text{gp120-CRA}$$

Fig 2: Reaction scheme for initial noncovalent binding of a CRA by gp120 and the subsequent covalent reaction. Hydrolysis of the covalent gp120-CRA complex is assumed to be negligible.

Gp120 derivitization at Lys side chains with the phosphonate diester and introduction of biotin at additional Lys side chains was done as in the preceding section. The resultant preparation, designated gp120-pCRA, displayed a propensity to form covalent oligomers upon incubation at 37°C in a neutral pH buffer (**Fig 1**). Under similar conditions, no oligomerization of control EGFR-pCRA or albumin-pCRA was observed. Electrophoresis of the reaction mixture under reducing conditions did not affect the mass of the oligomers, indicating that the S-S bonding is not a factor in oligomerization. Increasing oligomerization was observed with increasing derivitization of gp120 moles by the phosphonate diester moeities (**Fig. 1**). Taken together, these observations suggest that the oligomerization reaction occurs by covalent binding of gp120 nucleophile(s) with the phosphonate ester.

The chemical reactivity of gp120 is likely to derive from an amino acids that become activated by interaction with spatially neighboring residues. The activation is dependent on the three-dimensional folding pattern of the protein. For instance, proximity of Ser and His residues within hydrogen bonding distance imparts nucleophilic reactivity to the Ser oxygen atom and allows it to attack the electrophilic carbon in the peptide bond cleavage reaction. The phosphonate reactive site of gp120 can be mapped by forming adducts of the biotinylated hapten CRA Ib. The adduct is then reduced (2-mercaptoethanol) and alkylated (iodoacetamide) under denaturing conditions (6 M urea) and then digested with trypsin. Polypeptide fragments are separated by HPLC and fractions of interest are analyzed by mass spectroscopy (MALDI-TOF). Biotin-containing peptides from the HPLC are identified by a competitive ELISA in which streptavidin–peroxidase is allowed to bind biotinylated BSA immobilized in ELISA plates. Electrophoresis of the fractions on high-density polyacrylamide gels can be done to confirm the presence of phosphonylated peptides. It is important to track the presence of biotin containing peptides, because covalent bonds such as between the Ser hyroxyl and the phosphorus atom can be hydrolyzed unless appropriate precautions are implemented. The procedure allows determination of the peptide to which the CRA is bonded by comparison with the predicted mass of all peptide fragments generated by trypsin cleavage [at R/K-X bonds;. (expected mass of labeled peptide) = (mass of unlabeled peptide + mass of CRA – mass of the leaving group phenol)]. Accuracy is on the order of 1 amu. MS/MS analysis allows assignment of the phosphonate group to an individual residue within the peptide sequence.

2. Preparation of covalent gp120 oligomers. We believe that two distinct interactions underlie covalent self-assembly of gp120-CRA structures (**Fig. 2**) into the non-covalently guided trimer. gp120-CRA molecules first associate with each other noncovalently (like gp120). In the case of gp120, the oligomers are very unstable. Consequently, the predominant state of gp120 in physiological solutions is monomeric. Noncovalently associated gp120-CRA molecules, however, are stabilized by covalent bond formation between the phosphorus atom and the putative serine protease-like nucleophile in gp120. An important aspect of this model is correct spatial pairing of the electrophilic phosphorus with the nucleophile. This can only happen because this interaction simulates some natural electrophile-nucleophile pair responsible for intermolecular binding in gp120 oligomers devoid of phosphonate moieties. It is well known that gp41 facilitates gp120 oligomerization. In addition, it is known that gp120 monomers tend to interact with each other independent of a facilitatory role of gp41 [23]. Thus, it is reasonable to expect that the covalently assembled gp120 oligomers simulates the native structure of the protein (as a trimer on the virus surface). Biochemical characterization of the nucleophilicity of gp120 can help understand the covalent oligomerization process, e,g., to define the contribution of the specific noncovalent interactions between gp120 monomers. This is readily done by study of oligomerization at increasing concentrations of gp120 and irrelevant proteins mixed with the gp120-CRA preparation. Inhibition of the covalent oligomerization reaction by gp120 indicates a saturable process (as opposed to random covalent reactivity of the phoshonate group). Irrelevant proteins (e.g., albumin, EGFR) at similar concentrations, on the other hand, will no effect on the rate of covalent oligomerization. Acceleration of the covalent reaction by increasing concentrations of gp41 (intracytoplasmic tail removed) can be studied, and the reaction occurs more rapidly, this suggests that the gp120 protomers in the aggregate are interfaced to simulate the native trimeric structure of the protein, as gp41 is known to promote gp120 oligomerization.

Gp120-trimers stabilized by covalent linkages are shown in **Fig 3.** Structure **I** is designed based on self-oligomerization of gp120-pCRAs observed in Fig 1. Formation of this structure is guided by noncovalent association of gp120. Since the oligomerization of the gp120-pCRA is a spontaneous reaction, no special reagent is necessary for preparation of structure **I** (optimization of the reaction condition may be necessary to maximize yield of the trimer;). For synthesis of trimer **II**, a trivalent phosphonate cross-linker has been designed to permit the covalent assembly of gp120 (**Fig. 3**).

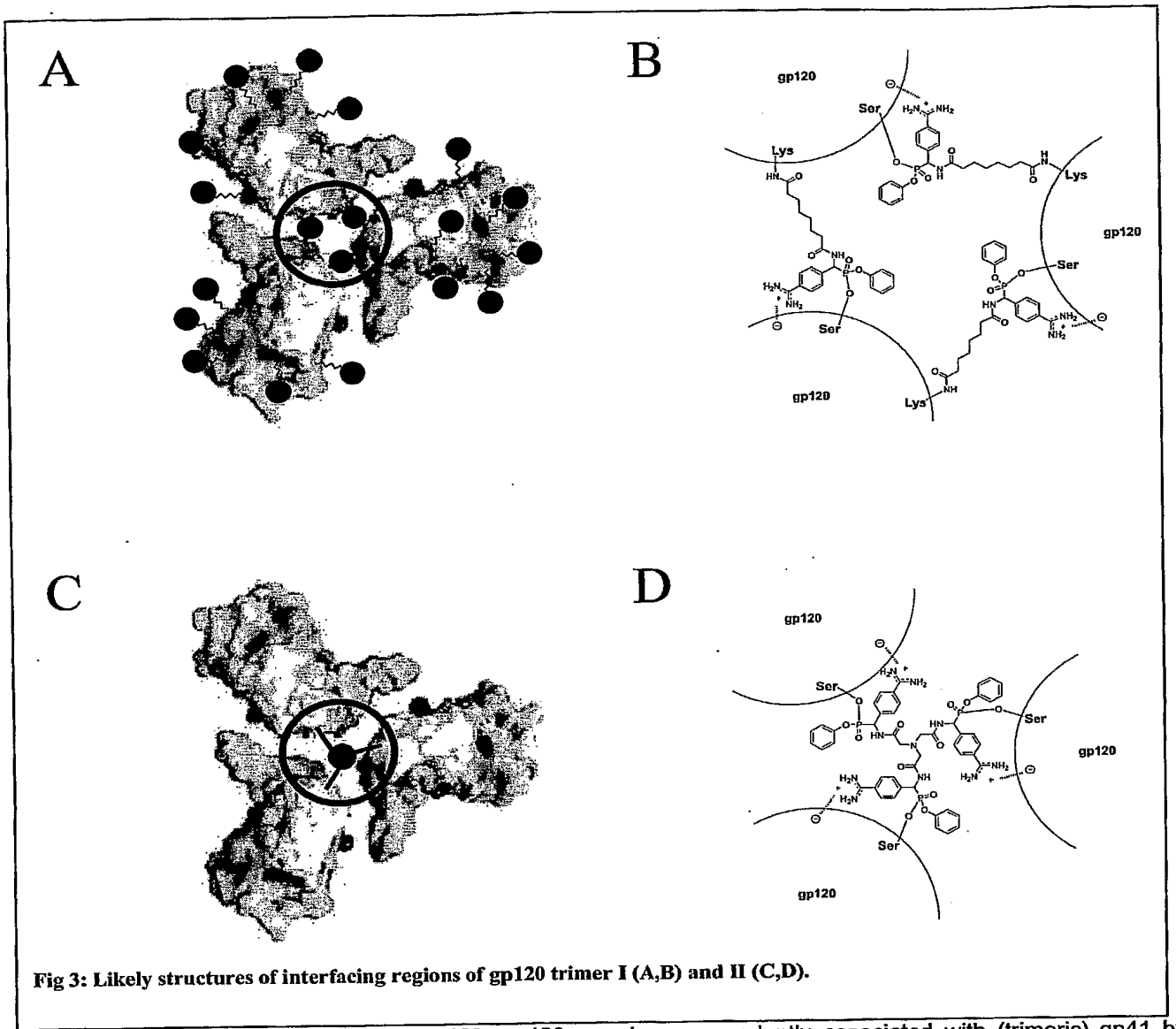

Fig 3: Likely structures of interfacing regions of gp120 trimer I (A,B) and II (C,D).

On the native virion, after cleavage of gp160, gp120 remains noncovalently associated with (trimeric) gp41 by noncovalent interactions (including hydrogen bonding). Published approaches to formation of gp120 trimers in solution, on the other hand, involve gp120 tandemly covalently linked to either trimerizing gp41 ectodomain or GCN4 or fibritin [21,22]. The resulting trimers may thus be prevented by these covalent associations from forming the fully native structure as occurs on the natural virion. Therefore, it is useful to compare the kinetics of formation of trimers from CRA-gp140 with that from CRA-gp120 trimer. More rapid formation of trimer from CRA-gp120 verifies the formation of a more native structure. Detailed kinetic studies of the oligomerization process can be done to determine the rate constants of dimerization and trimerization. An independent test of the importance of noncovalent forces in guiding correct self-assembly of the gp120-pCRA consists of Ab binding studies described below, in which **I** (**Fig. 3**) should bind better than **II** to Abs known to recognize the native trimeric structure of gp120. Covalent assemply of the latter molecule (**II**) is an artificial process, as it is constrained more by the topography of the trielectrophile used to achieve trimerization than by any natural assembly processes. There is no reason to believe that this molecule should show improved reactions with the Abs compared to monomer gp120, except purely for coincidental reasons. Further enzymology studies are done as needed to characterize the active site nucleophile further (e.g., pH dependency studies – a neutral pH optimum is indicative of a serine protease-like nucleophile; residues such as Lys display greater nucleophilic reactivity at extreme alkaline pH).

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3. Validation of native trimer structure: The extent to which the covalent trimer structure simulates native trimeric gp120 CRA is measured through its interaction with well characterized Mabs know to recognize native HIV envelope, including , Mab IgG1b12, 2G12 and Mab S1-1. ELISA and BiaCore techniques are used to measure the binding strength of the interaction. By capturing the antibodies on immobilized antigen, one obtains a biologically relevant measure of [multivalent] *avidity*. Comparison of the trimer reactivity is with gp120 monomer (devoid of phosphonate moieties) as well as monomer gp120-CRA VIb (freshly thawed from its frozen state to ensure that it exists predominantly in the monomer form; the gp120-CRA oligomerizes appreciately over in several hours after it is thawed). Covalent binding of the trimer by the antibodies is possible, as the intermolecular reaction between the gp120-CRA monomers requires consumption of only one phosphonate moiety per molecule, and several additional phosphonates are available for the reactions with antibodies. Comparison of the control monomer gp120-CRA and covalent trimers will allow assessment of both components of the reaction with the antibodies, that is, the noncovalent and the covalent component. Another available strategy is to allow spontaneous hydrolysis of the excess phosphonates in the trimer at mild alkaline pH for a few hours – the spontaneous cleavage of ester bonds generates phosphonic acid moieties that do not express appreciable covalent reactivity. Antibody binding by such a preparation should occur primarily by noncovalent means. The ELISA studies are conducted without and with inclusion of an SDS treatment step prior to development of the reaction with the second antibody. This approach has been previously used as the means to distinguish between the covalent and noncovalent binding components. Regardless of whether the antibody binding to the trimer is covalent or noncovalent, the interpretation remains unchanged, that is, if the covalent trimer binds the Abs better than the monomer gp120/monomer gp120-CRA, the trimer must offer superior antibody epitopes.

4. Raising and analyzing Abs to covalently stabilized trimer: Polyclonal antibodies to the covalently stabilized trimers will be raised in Balb/C mice. Groups of 5 mice each are immunized with trimer and (as a control) monomer, respectively. The resulting polyclonal sera are analyzed for neutralization of primary isolates of HIV using peripheral blood mononuclear cells. Simultaneously IgG is purified from the sera of immunized mice (by protein G-Sepharose chromatography) and characterized by ELISA using the covalent trimer as the antigen. Monoclonal antibodies are prepared by standard hybridoma methods and screened for binding to immobilized trimers. These are characterized by HIV neutralization assays. As I contains exposed unreacted phosphonates, it might provoke formation of catalytic antibodies. BiaCore SPR analysis is done to determine irreversibility (koff should approach zero). Covalent ELISA, SDS-electrophoresis and catalysis assays are also carried out using standard methods. The monoclonal Abs can be prepared from mice expressing human antibody genes as needed to facilitate passive immunotherapy applications.

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Examples of sites of attachment of electrophile Y ~~examples~~ to peptides and proteins (Lys residues)

exEGFR (GenBank#P00533):

```
1   LEEKKVCQGT SNKLTQLGTF EDHFLSLQRM FNNCEVVLGN LEITYVQRNY DLSFLKTIQE
61  VAGYVLIALN TVERIPLENL QIIRGNMYYE NSYALAVLSN YDANKTGLKE LPMRNLQEIL
121 HGAVRFSNNP ALCNVESIQW RDIVSSDFLS NMSMDFQNHL GSCQKCDPSC PNGSCWGAGE
181 ENCQKLTKII CAQQCSGRCR GKSPSDCCHN QCAAGCTGPR ESDCLVCRKF RDEATCKDTC
241 PPLMLYNPTT YQMDVNPEGK YSFGATCVKK CPRNYVVTDH GSCVRACGAD SYEMEEDGVR
301 KCKKCEGPCR KVCNGIGIGE FKDSLSINAT NIKHFKNCTS ISGDLHILPV AFRGDSFTHT
361 PPLDPQELDI LKTVKEITGF LLIQAWPENR TDLHAFENLE IIRGRTKQHG QFSLAVVSLN
421 ITSLGLRSLK EISDGDVIIS GNKNLCYANT INWKKLFGTS GQKTKIISNR GENSCKATGQ
481 VCHALCSPEG CWGPEPRDCV SCRNVSRGRE CVDKCNLLEG EPREFVENSE CIQCHPECLP
541 QAMNITCTGR GPDNCIQCAH YIDGPHCVKT CPAGVMGENN TLVWKYADAG HVCHLCHPNC
601 TYGCTGPGLE GCPTNGPKIP S
```

exEGFR has 36 lysines at the following position: 4, 5, 13, 56, 105, 109, 165, 185, 188, 202, 229, 237, 260, 269 270, 301, 303, 304,311, 322, 333, 336, 372, 375, 407, 430, 443, 454, 455, 463, 465, 476, 504, 569, 584, 618.

Prothrombin (Genbank# P00734):

```
1   QHVFLAPQQA RSLLQRVRRA NTFLEEVRKG NLERECVEET CSYEEAFEAL ESSTATDVFW
61  AKYTACETAR TPRDKAACLE GNCAEGLGTN YRGHVNITRS GIECQLWRSR YPHKPEINST
121 THPGADLQEN FCRNPDSSTT GPWCYTTDPT VRRQECSIPV CGQDQVTVAM TPRSEGSSVN
181 LSPPLEQCVP DRGQQYQGRL AVTTHGLPCL AWASAQAKAL SKHQDFNSAV QLVENFCRNP
241 DGDEEGVWCY VAGKPGDFGY CDLNYCEEAV EEETGDGLDE DSDRAIEGRT ATSEYQTFFN
301 PRTFGSGEAD CGLRPLFEKK SLEDKTEREL LESYIDGRIV EGSDAEIGMS PWQVMLFRKS
361 PQELLCGASL ISDRWVLTAA HCLLYPPWDK NFTENDLLVR IGKHSRTRYE RNIEKISMLE
481 KIYIHPRYNW RENLDRDIAL MKLKKPVAFS DYIHPVCLPD RETAASLLQA GYKGRVTGWG
541 NLKETWTANV GKGQPSVLQV VNLPIVERPV CKDSTRIRIT DNMFCAGYKP DEGKRGDACE
601 gdsggpfvmK spfnnrwyqm givswgegcd rdgKygfyth vfrlKKwiqK vidqfge
```

Prothrombin has 29 lysines at the following position:29, 62, 75, 114, 218, 222, 254, 319, 320, 325, 359, 390, 403, 415, 481, 502, 504, 505, 533, 543, 552, 572, 589, 594, 610, 634, 645, 646, 650

Gp120MN(protein science, numbering include peptide signal GenBank # M17449 with the following 6 mutations):

(30G:T, 71H:Q, 79D:N, 171G:R, 365T:N, 465D:N)

```
28  IPGEKLWVTV YYGVPVWKEA TTTLFCASDA KAYDTEVHNV WATHACVPTD PDPQEVELVN
88  VTENFNMWKN NMVEQMHEDI ISLWDQSLKP CVKLTPLCVT LNCTDLRNTT NTNNSTANNN
148 SNSEGTIKGG EMKNCSFNIT TSIGDKMQKE YALLYKLDIV SIDNDSTSYR LISCNTSVIT
208 QACPKISFEP IPIHYCAPAG FAILKCNDKK FSGKGSCKNV STVQCTHGIR PVVSTQLLLN
268 GSLAEEEVVI RSENFTDNAK TIIVHLNESV QINCTRPNYN KRKRIHIGPG RAFYTTKNII
328 GTIRQAHCNI SRAKWNDTLR QIVSKLKEQF KNKTIVFTQS SGGDPEIVMH SFNCGGEFFY
388 CNTSPLFNST WNGNNTWNNT TGSNNNITLQ CKIKQIINMW QEVGKAMYAP PIEGQIRCSS
448 NITGLLLTRD GGKDTDTDDT EIFRPGGGDM RDNWRSELYK YKVVTIEPLG VAPTKAKRRV
508 VQREKR
```

Gp120MN has 35 lysines at position: 32, 45, 58, 96, 116, 120, 155, 209, 173, 176, 183, 212, 232, 236, 237, 241, 245, 287, 308, 310, 324, 341, 352, 354, 358, 360, 419, 421, 432, 460, 477, 479, 502, 504, 512.

VIP:

HSDAVFTDNYTRLRKQMAVKKYLNSIN

VIP has 3 lysines at the following positions:15, 20, 21

p53 (GenBank# BAC57998):

```
  1  MGARSGARGA LLLALLLCWD PRLSQAGTDS GSEVLPDSFP SAPAEPLPYF LQEPQDAYIV
 61  KNKPVELRCR AFPATQIYFK CNGEWVSQND HVTQEGLDEA TGLRVREVQI EVSRQQVEEL
121  FGLEDYWCQC VAWSSAGTTK SRRAYVRIAY LRKNFDQEPL GKEVPLDHEV LLQCRPPEGV
181  PVAEVEWLKN EDVIDPTQDT NFLLTIDHNL IIRQARLSDT ANYTCVAKNI VAKRRSTTAT
241  VIVYVNGGWS SWAEWSPCSN RCGRGWQKRT RTCTNPAPLN GGAFCEGQAF QKTACTTICP
301  VDGAWTEWSK WSACSTECAH WRSRECMAPP PQNGGRDCSG TLLDSKNCTD GLCMQNKKTL
361  SDPNSHLLEA SGDAALYAGL VVAIFVVVAI LMAVGVVVYR RNCRDFDTDI TDSSAALTGG
421  FHPVNFKTAR PSNPQLLHPS VPPDLTASAG IYRGPVYALQ DSTDKIPMTN SPLLDPLPSL
481  KVKVYSSSTT GSGPGLADGA DLLGVLPPGT YPSDFARDTH FLHLRSASLG SQQLLGLPRD
541  PGSSVSGTFG CLGGRLSIPG TGVSLLVPNG AIPQGKFYEM YLLINKAEST LPLSEGTQTV
601  LSPSVTCGPT GLLLCRPVIL TMPHCAEVSA RDWIFQLKTQ AHQGHWEEVV TLDEETLNTP
661  CYCQLEPRAC HILLDQLGTY VFTGESYSRS AVKRLQLAVF APALCTSLEY SLRVYCLEDT
721  PVALKEVLEL ERTLGGYLVE EPKPLMFKDS YHNLRLSLHD LPHAHWRSKL LAKYQEIPFY
781  HIWSGSQKAL HCTFTLERHS LASTELTCKI CVRQVEGEGQ IFQLHTTLAE TPAGSLDTLC
841  SAPGSTVTTQ LGPYAFKIPL SIRQKICNSL DAPNSRGNDW RMLAQKLSMD RYLNYFATKA
901  SPTGVILDLW EALQQDDGDL NSLASALEEM GKSEMLVAVA TDGDC
```

p53 has 35 lysines at the following postions: 61, 63, 80, 140, 153, 162, 189, 228, 233, 268, 292, 310, 346, 357, 358, 427, 465, 481, 483, 576, 586, 638, 693, 725, 743, 748, 769, 773, 788, 809, 857, 865, 886, 900, 932

IL-1 alpha, (GenBank# P01583):

```
  1 APFSFLSNVK YNFMRIIKYE FILNDALNQS IIRANDQYLT AAALHNLDEA VKFDMGAYKS
 61 SKDDAKITVI LRISKTQLYV TAQDEDQPVL LKEMPEIPKT ITGSETNLLF FWETHGTKNY
121 FTSVAHPNLF IATKQDYWVC LAGGPPSITD FQILENQA
```

IL-1 alpha has 11 lysines at the following positions: 10, 18, 52, 59, 62, 66, 75, 92, 99, 118, 134

IL1- beta (GenBank# P01584):

```
  1 APVRSLNCTL RDSQQKSLVM SGPYELKALH LQGQDMEQQV VFSMSFVQGE ESNDKIPVAL
 61 GLKEKNLYLS CVLKDDKPTL QLESVDPKNY PKKKMEKRFV FNKIEINNKL EFESAQFPNW
121 YISTSQAENM PVFLGGTKGG QDITDFTMQF VSS
```

IL-1 beta has 15 lysines at position: 16, 27, 55, 63, 65, 74,77,88, 92, 93,94, 97, 43, 109, 138